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AN OUTBREAK OF AN ABERRANT TYPE OF RINDERPEST IN TANGANYIKA TERRITORY

By

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Tanganyika Territory

INTRODUCTION

An outbreak of disease in cattle in the Tabora District of the Western Province of Tanganyika Territory occurred in December, 1944. Although simulating rinderpest, its manifestations were of such an unusual type that a full field and laboratory investigation was instituted. The area in question had been free of this disease for at least six years previously. The chief type of cattle involved was an Ankole Zebu-cross, an animal which is regarded as being very highly susceptible to rinderpest. In this connection it is of interest to note that in a test carried out by one of us (H. J. L.) in which similar cross-bred cattle were injected with Kenya attenuated goat virus, a mortality of about 70 per cent. resulted. It can be taken, therefore, that the susceptibility of the large majority of the cattle involved was so high that in an outbreak of the ordinary character in this area one would expect to find the typical symptoms of rinderpest in an easily detectable form.

It has been well known for many years that the clinical symptoms produced by rinderpest virus vary in severity, but it was thought that this phenomenon was due mainly to differences in the resistance of the hosts rather than to any variation in the virulence of the virus. In this case, however, the circumstances mentioned above made it one of exceptional interest and the details are accordingly set out at some length.

The outbreak was first reported on December 5th, two miles north of Tabora town, in a herd of 70 first-class Ankole-Zebu cattle, which were, therefore, highly susceptible to rinderpest. On examination a considerable number were well on the way to recovery. Many were free from any signs of diarrhoea and did not exhibit the emaciated condition usually associated with the terminal stages of rinderpest. The herdsman said that the disease had been in existence quite a time and that many cattle had already recovered.

CLINICAL INVESTIGATION

The general condition of the herd was poor and the animals were heavily tick-infested. Most of the cattle in poorer condition had suffered from an attack of the disease and many were recovering from it. The main symptoms were watery or muco-purulent discharges from the eyes, and spreading and irregular ulcers in the

mouth, on the mucous membrane at the commissures of the lips and in front of the molar teeth. To a lesser extent ulcers appeared on the upper lip and the gums, but were seldom found on the inner aspect of the under lip. The majority of the ulcers were clean, showing reddened surfaces and edges, and in those which contained debris, this was easily brushed away leaving a surface which bled easily but slightly. No objectionable smell was noted and no drooling occurred from the mouths of infected animals.

Diarrhoea was not a feature of the disease, and although some animals passed fairly fluid faeces, probably as the result of ingesting fresh grass, there was no evidence of the foetid liquid faeces so often observed in typical rinderpest. A fairly high fever was registered in affected animals.

The superficial glands did not appear to be involved and gland and blood smears revealed no parasites. Streptothricosis was present, but was of a mild type except in one or two very old animals.

The mortality rate was low. Up to the time of discovery of the outbreak, three animals in the herd had died (4·3 per cent.); these deaths, however, may have been affected by the onset of the rains.

It was considered that East Coast fever, trypanosomiasis, and indeed all the protozoal diseases, could be ruled out. Other conditions which suggested themselves were foot-and-mouth disease, three-day sickness, heartwater (Rickettsia), bluetongue and plant poisoning. The syndrome, however, did not fit satisfactorily with any of these diseases, although in the early stages the outbreak showed some similarity to bluetongue in cattle, in that the mouth lesions closely resembled those described by Bekker *et al.* (1934) in cattle affected with this disease.

Blood taken from infected animals was inoculated into sheep and goats without producing any reaction. At the same time citrated blood and various organs (spleen, lymph gland, abomasum, ileum, caecum, and rectum) were forwarded to the laboratory for examination and test.

In the meantime the disease continued to spread, though slowly and in jumps. The first extension was reported on December 15th in a herd of 50 at Simbo some four miles from the original focus. Here the symptoms were even milder, though young animals seemed to be more severely affected than the older stock. Most of the animals had already had the disease by the time the outbreak was discovered, although five head were still sick and two had died (4 per cent.). No evidence could be found that any stock had been brought into the area recently, nor was there any possible bovine link between these outbreaks and any other area where rinderpest was known to exist.

Further specimens from a beast with a temperature of 104·8° F. were sent to the laboratory from this second centre.

A third outbreak was reported on December 21st in an area three miles away from the second and some seven miles from the

first. The symptoms looked more like those of rinderpest since there was a more typical nasal discharge. A fourth focus came to light on December 22nd at a point about four miles south-west of Tabora town (all the others were north of Tabora) and with the more characteristic symptoms which were observed in the last two foci no difficulty was experienced in arriving at a diagnosis of rinderpest. Further, several of the recovered cattle in the first and second outbreaks inoculated with Kenya attenuated goat virus failed to react whereas animals which had not been affected by the disease reacted typically.

Although the disease had been in existence for at least three weeks, in each outbreak cattle in kraals 100 yards away from the affected herds escaped infection.

Even after the most intensive comb-out of the whole area no evidence was obtained to indicate that the infection had been introduced by illicit movement of stock or by any other means ; nor were there any reports of sickness or mortality in game in the region affected.

At this stage it was considered that an anti-rinderpest campaign to embrace the whole of Tabora district was called for and the operations were put in hand immediately.

LABORATORY INVESTIGATION

The first material received at the laboratory consisted of formolised specimens, and blood and other tissues preserved in citrate and glycerine respectively, from a beast which had died of the disease. The abomasum showed inflammation in the fundus with ulcers in the pyloric region. Portions of the large intestine presented no lesions other than a slight inflammation at the ileo-caecal valve and longitudinal haemorrhagic streaks in the mucosa of the rectum.

The main object of the investigation was to prove whether this disease, although atypical, was rinderpest, and the following tests were therefore made.

A group of sheep, goats and oxen, believed to be susceptible to rinderpest, a bush pig known to be immune to rinderpest, and guinea-pigs were used. No parasites were found in their blood. Ox No. 108, goat No. 13 and sheep No. 1 received a subcutaneous injection of 5 c.c. of citrated blood drawn from a yearling calf which was showing a temperature of 103·8° F.

Ox No. 109, goat No. 14 and sheep No. 2 received a subcutaneous injection of spleen pulp from a calf which had died of the disease at Tabora. Ox No. 110, goat No. 15, and sheep No. 3 received a subcutaneous injection of gland pulp from the same calf.

A part of the same sample of blood as was given to ox No. 108 was injected subcutaneously into the bush pig and intradermally into the pads of the hind feet of two guinea-pigs, Nos. 28 and 29. Two more guinea-pigs, Nos. 30 and 31, were injected intradermally

with juice from the calf spleen referred to above. No reaction, thermal or clinical, occurred in any of these animals until the morning of December 19th, 1944, *i.e.*, on the seventh day after the inoculations, when ox No. 110 showed a temperature of 103° F., followed by a full thermal reaction lasting six days and attaining a peak of 104·6° F. Fourteen days after the commencement of this reaction, ox No. 110 was challenged with rinderpest virus and showed no reaction.

None of the other animals showed any reaction whatever to the original inoculation but both the remaining oxen (*viz.*, Nos. 108 and 109) reacted typically to the subsequent challenge with rinderpest virus.

The thermal reaction displayed by ox No. 110 is shown in Table I.

TABLE I

OX 110

	December, 1944										
	13	14	15	16	17	18	19	20	21	22	23
Days after inoculation ...	1	2	3	4	5	6	7	8	9	10	11
Temperature	100·4	100·8	101·4	101·2	101	102·6	103	104	104·6	104·4	103·6

	24	25	26	27	28	29	
Days after inoculation ...	12	13	14	15	16	17	
Temperature	102	101·6	102·4	101·6	101	100·8	and thereafter normal

The symptoms shown by ox No. 110 were as follows. On the tenth day, erythema of the gums round the corner incisors was apparent. The animal showed a general dullness and the characteristic odour associated with rinderpest was faintly discernible in the breath. On the 11th day, diarrhoea was evident and a slight mucous discharge appeared in the right nostril. Small ulcers had developed on the gums round the corner incisors. These symptoms, however, rapidly abated and the animal returned to normal health.

On the first day of its thermal reaction blood was drawn from ox No. 110 into citrate solution and injected into two more oxen believed to be susceptible to rinderpest (Nos. 111 and 112), and into two sheep (Nos. 4 and 5). The two oxen started to react thermally on the fifth and fourth days respectively. The reactions in both cases attained a peak of 105·4° F. and lasted for five and six days respectively. Symptoms were mild, although slight ulcer formation occurred in the mouths of both oxen. Both were immune to subsequent challenge with rinderpest virus. Neither of the sheep showed any noticeable reaction.

In view of the results of the first laboratory passage and the apparent absence of virulence for sheep, it was felt at this stage that bluetongue could be ruled out and subsequent tests were directed towards confirmation of the belief, which was now fairly firmly established, that the disease was in fact rinderpest. A third passage was made from the pooled bloods of oxen Nos. 111 and 112 drawn when these were reacting thermally (eighth day). Passaging was continued until a further five transfers had been completed. The history of this virus from its receipt at the laboratory to its eighth passage is shown in Table II.

A variation in procedure was introduced at the fourth passage, when a portion of the blood drawn from the two reacting oxen, Nos. 119 and 120, was filtered through a Seitz bacterial filter and the plasma filtrate injected into two oxen, Nos. 121 and 122. Two other oxen, Nos. 123 and 124, were inoculated with the whole blood.

Nos. 121 and 122 did not react. Nos. 123 and 124 reacted in the usual way, No. 123 attaining a peak of 103·6° F. and maintaining a thermal response from the sixth to the tenth days, and No. 124 attaining a peak of 105° F. and reacting from the fifth to the 12th days. Both these oxen showed characteristic but mild symptoms of rinderpest, and both were immune to subsequent virus challenge.

Blood taken from oxen Nos. 123 and 124 at the height of their thermal reaction was shaken very thoroughly with sterile glass beads and was also passed through a Seitz filter. The filtrate, injected into oxen Nos. 121 and 122, evoked a thermal response. No. 121 reacted from the sixth to the 14th days and showed quite severe symptoms of rinderpest; it proved immune to a subsequent challenge with rinderpest virus. No. 122 reacted, but its reaction was delayed (10th to 17th days). The beast was very severely affected and was slaughtered on the 18th day after the second filtrate injection. (See below.)

The results with (*a*) blood undisturbed by shaking before filtration; and (*b*) blood filtered after vigorous agitation, were not incompatible with the belief that the pathogen was the virus of rinderpest, as it is known that this virus is very firmly attached to the white cells. (Daubney, 1927; Evans, 1927; Hornby, 1928.) The virulence of whole rinderpest blood is probably about 200 times greater than that of the filtrate.

By the time the eighth passage was reached the infected animals were showing a syndrome typical of moderately severe rinderpest. The most seriously affected animal was No. 122, which was slaughtered in a very sick condition, 18 days after inoculation. *Post-mortem* examination revealed diffuse gastritis, especially marked in the fundus of the abomasum. There were a few areas of erosion with a yellow fibrinous covering to some of them and a small haemorrhagic ulcer ¼ in. in diameter near the pylorus. Scattered petechiae and slight inflammation were present in the duodenal mucosa, and in the caecum and colon a few petechiae were seen. All the other organs were apparently normal.

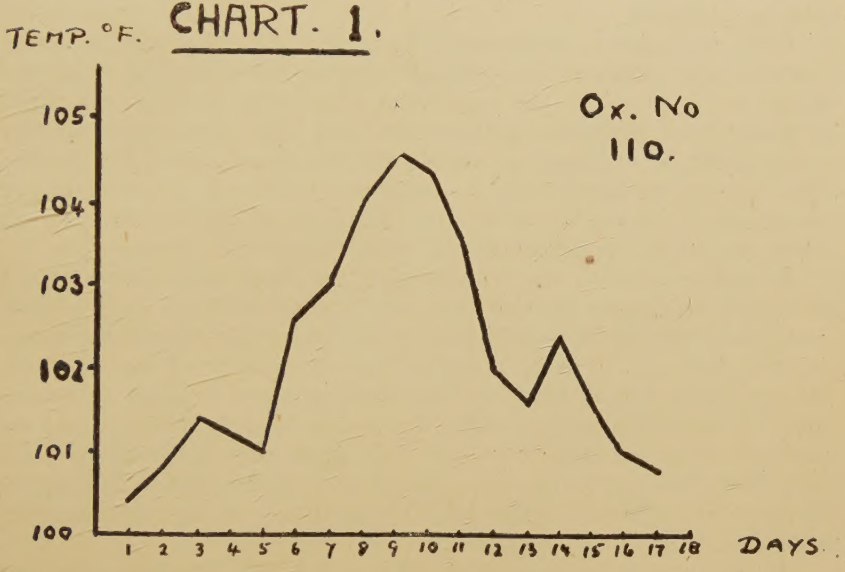
TABLE II

PASSAGE OF VIRUS

Passage	*Oxen*	*Thermal peak*	*Commencement of thermal reaction*	*Termination thermal reaction*	*Duration of thermal reaction*	*Remarks regarding symptoms*
1	110	104·6° F.	7th day	12th day	6 days	Mild symptoms. Diarrhoea. Slight nasal discharge. Small ulcers on gums round corner incisors.
2	111	105·4° F.	5th day	10th day	6 days	Mild symptoms. Breath faintly foetid. No diarrhoea. Slight nasal and ocular discharge. Small ulcer on hard palate (III). Small ulcer behind corner incisors (112).
	112	105·4° F.	4th „	10th „	7 „	
3	119	104·6° F.	4th day	9th day	6 days	Mild symptoms. Slight nasal discharge. No diarrhoea but slightly softened faeces with a little blood.
	120	103·6° F.	5th „	7th „	3 „	
4	121	No reaction—filtered blood used				
	122	„ — „ „				
	123	103·6° F.	6th day	10th day	5 days	Symptoms more pronounced. Foetid breath. Erythema and ulceration of gums and cheek papillae. Nasal and ocular discharge. Diarrhoea. Slight streptothricosis, cough.
	124	105° F.	5th „	12th „	8 „	
5	121	105° F.	6th day	14th day	9 days	Blood filtered after agitation. Typical symptoms of fairly severe rinderpest.
	122	105° F.	10th „	17th „	8 „	
	128	104·6° F.	6th „	10th „	5 „	Mild symptoms. Erythema of gums and fraenum linguae.
	129	105° F.	5th „	10th „	6 „	Ulceration of papillae. Ocular and nasal discharges.
6	132	105·6° F.	5th day	12th day	8 days	Mild symptoms, similar to those shown by Ox in first passage.
	134	104·6° F.	5th „	10th „	6 days	„ „ „ „
7	135	105·4° F.	4th day	12th day	9 days	Symptoms a little more severe than those of Nos. 132 and 134. Typical of rinderpest. No 135, symptoms complicated by presence of *T. theileri*.
	136	103·6° F.	4th „	7th „	4 „	
8	155	105° F.	4th day	10th day	7 days	Symptoms typical of fairly severe rinderpest.
	156	104·8° F.	4th „	8th „	5 „	„ „ „ „

Apart from ox No. 122, all the oxen which were infected during these passages recovered and were found subsequently to be immune to rinderpest.

After the eighth passage, economy in the use of susceptible oxen necessitated the storage of virulent blood in the refrigerator between passages. However, after a storage of nine days, the blood failed to infect oxen. It is rather surprising that the virus should be lost during such a short period of storage. It is possible that factors at present unknown played a part in its destruction. In our experience



virulent rinderpest blood loses its virulence more rapidly than does spleen tissue, but it is generally possible to keep virulent blood in the refrigerator for at least one month without its infectivity being seriously impaired. During the course of this investigation, samples of blood were received at the laboratory from the field outbreak. Although they were sent on ice in thermos flasks, most of them failed to infect susceptible oxen. The virus from this outbreak, therefore, appears to have been very fragile.

DISCUSSION

The laboratory investigation leaves no doubt in our minds that the disease under examination was rinderpest, although both in the field and in the laboratory it had not followed the course which is normally expected. The outstanding features of the outbreak were the extraordinary mildness of the disease, especially when it first

appeared in the field among stock which experience has shown to be very highly susceptible, and the epizootiology of the outbreak. No link whatever could be found between this and any other outbreaks of rinderpest.

As the disease progressed by natural passage in the field, the virulence appeared to increase and the syndrome more closely resembled the ordinary manifestation of rinderpest. It is obvious, therefore, that at its first appearance in the Tabora outbreak, the virus involved was in a very attenuated state. Any suggestions as to the manner in which this attenuation had been brought about must be pure speculation, but the following theoretical considerations may be advanced.

The attenuation must have taken place in animals other than cattle as no history of any disease simulating rinderpest could be traced in cattle within the area affected over the previous six years at least. It is obvious that the virus must have been maintained in living hosts, as its fragility outside the host was apparently greater than that of the usual form of rinderpest virus. It is reasonable to assume that the carrier hosts were members of one or more of the many species of the game animals which abound in Tanganyika.

It would seem to the authors that the virus may have adapted itself to life in some game animal and had passed from host to host without having the opportunity over a considerable period of returning to cattle. It is quite possible that attenuation of the virus for the bovine host took place during these passages to such an extent that a re-establishment in cattle became extremely difficult and no outbreak occurred until a contact was made with cattle of particularly high susceptibility, *viz.*, the Ankole-Zebu cattle of Tabora. Having once obtained a foothold, the virulence of the virus rapidly became enhanced by passage.

It is of interest that in ox No. 135 (see Table II) the virus appeared to exacerbate a latent infection with *Trypanosoma theileri* producing a swarming infection. The symptoms were severe and resembled true rinderpest. This observation agrees with past experience in this laboratory on an association of rinderpest with *T. theileri* infection (Hornby *et al.*, 1934) and with similar findings in Uganda (Carmichael, 1926).

SUMMARY

Virus of an outbreak of a disease simulating very mild rinderpest was recovered from a lymphatic gland of an affected calf. It caused a reaction in an ox when it was passaged by blood inoculation through a further series of oxen.

The virus evoked a thermal reaction similar to that of rinderpest. The symptoms shown by the affected animals were not strictly constant, but the general picture of the disease was as follows. The incubation period varied from four to seven days and the duration of the thermal reaction from three to nine days. There was general

dullness during and shortly after the reaction and a nasal discharge of a serous nature, becoming purulent in some cases. Conjunctivitis, erythema of the gums and fraenum linguae and ulceration of the gums, fraenum linguae, buccal papillae, mucosa of the tongue, lips and occasionally the muzzle occurred in several affected animals. There was a little diarrhoea, but it was never severe.

As a result of serial passages, there was a tendency towards a shortening of the incubation period and a slight increase in the general severity of the disease, although after eight passages the infection never became what might be described as a "killing" disease. All the cattle which reacted to the virus were subsequently immune to rinderpest.

Filtered blood after vigorous agitation was infective, while blood filtered without agitation failed to infect. The virus appeared to be unduly fragile.

The apparent exacerbation of a latent *T. theileri* infection in one case by the virus is described.

ACKNOWLEDGMENTS

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BACTERIOLOGICAL FINDINGS AT *POST-MORTEM* EXAMINATION OF PIGS IN PALESTINE

By

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INTRODUCTION

Prior to 1939 the number of pigs in Palestine was small. The economic importance of the pig was slight compared with that of the other domestic animals, and the Veterinary Department was seldom called upon to deal with pig diseases. With the outbreak of war, however, and the entry into Palestine of large numbers of troops, a demand for pork and bacon was created that could only be met by a great increase in pig-breeding. Cases of disease among pigs were reported to the Veterinary Department more frequently than hitherto and the number of pigs sent to the laboratory for *post-mortem* examination accordingly increased.

In this paper a summary is made of the bacteriological findings in 100 pigs during the period October, 1944, to February, 1946. This has been done because, to the best of the writers' knowledge, no paper has hitherto been published giving details of micro-organisms likely to be encountered during routine examination of pigs in Palestine. The appearance of swine fever among the suddenly increased pig population was both anticipated and feared. The disease was first diagnosed in the country in April, 1939 and was immediately made notifiable. At that time only a very few pigs were involved and the disease disappeared almost immediately, but it reappeared in October, 1944. Since that time it has been present almost continuously and has caused losses sufficiently severe to constitute the major disease of pigs in the country and a constant source of danger to the pig industry.

Since no other pig disease in any degree comparable in severity with swine fever is at present known to exist in Palestine, little importance has been attached to occasional deaths due to other causes. As many cases of swine fever are notoriously difficult to diagnose, every *post-mortem* examination yielding the slightest degree of evidence of this disease was considered "suspicious for swine fever"; in the field the case was assumed to be one of swine fever until further *post-mortem* examinations, clinical considerations and subsequent developments in the piggery made a definite diagnosis possible.

The descriptions of the pathological changes noted *post-mortem* have been reduced to a minimum and the bacteriological findings have been summarised in tabular form.

MATERIALS AND METHODS

Most of the 100 *post-mortem* examinations were made in the Tel-Aviv laboratory, but a few were carried out in the field and the organs brought to the laboratory for bacteriological examination. In all cases referred to in this paper " *post-mortem* examination " means examination of the entire pig ; cases in which organs only were available are not included. Smears were prepared directly from the lung, heart, liver, spleen and kidney and stained by Giemsa and by Gram's method.

Material from the same organs was inoculated into serum broth, on to ox-blood agar and McConkey plates and into Hibler's brain-and-liver medium. Pure cultures of the various organisms isolated were studied as to their morphological, cultural and biochemical properties.

The Salmonellas isolated were typed by using the rapid slide agglutination technique (Kaufmann, 1939). Pathogenicity tests in mice were carried out by intraperitoneal inoculation of 0·3 ml. of a 24-hour broth culture and inoculated animals were kept under observation for at least one month. Smears and cultures were made from those which died.

MICRO-ORGANISMS ISOLATED FROM CASES OF SWINE FEVER

Before proceeding to discuss the organisms isolated from cases classed as swine fever, it is necessary to explain briefly the basis on which the diagnosis was made.

The first suspected case was encountered in October, 1944, after the disease had apparently been absent from the country for five years. Material was injected into two healthy pigs, obtained from the Government Farm ; one was inoculated subcutaneously with 10 ml. citrated whole blood and the other with a Berkefeld N filtrate of a saline suspension of liver, spleen and kidney (6 ml. intravenously and 3 ml. subcutaneously). The former pig died suddenly five days later without showing any clinical symptoms. *Post-mortem* examination revealed slight congestion of the mesenteric lymph glands, one small sub-epicardial haemorrhage and a few small subpleural haemorrhages on the apical lobes of the lungs. Bacteriological examination was negative. The other pig showed a febrile reaction lasting from the fourth to the seventh day after inoculation, and during this time was obviously sick. It then appeared to recover completely and when destroyed for *post-mortem* examination on the 12th day showed extensive " button " ulceration in the caecum and upper part of the colon. Bacteriological examination was negative. The results of these experiments, together with the original *post-mortem* findings and clinical information from the field, were accepted as sufficient proof of the presence of swine fever.

In almost all of the subsequent cases diagnosis was based entirely on history, clinical symptoms and *post-mortem* findings. The frequency distribution of the lesions found in 30 cases of swine fever uncomplicated by the presence of organisms of known pathogenic importance was as follows : inflammation and/or haemorrhage of lymphatic glands (23 cases) ; inflammation of the small intestine (20) ; haemorrhages in the lungs (20), kidneys (17) ; inflammation

and/or haemorrhage of gastric mucosa (17); haemorrhages in the sub-epicardium and/or sub-endocardium (15); infarcts of the spleen (12); haemorrhages in the subcutis (nine); "button" ulcers of the large intestine, notably caecum (five); haemorrhages in the epiglottis (three). In occasional acute cases, histological examination of the brain was made and the finding of an acute non-purulent encephalitis in some, but not all, of the cases examined helped in establishing a diagnosis. Negative bacteriological findings assisted in confirming the final diagnosis of swine fever.

Muir (1943) was unsuccessful in transmitting the virus of swine fever to laboratory animals and has summarised the literature on this subject. In Palestine attempts to infect mice, guinea-pigs and rabbits by intracerebral inoculation of a Berkefeld N filtrate of a saline suspension of liver/spleen/kidney from a typical case of swine fever from which no pathogenic organism was isolated, were also uniformly negative.

Swine fever in Palestine appears to resemble the disease as described in Europe and America though its exact relationship to the condition in these countries has not been determined by cross-immunity tests. On the other hand, it seems to differ from the disease in South Africa as described by De Kock *et al.* (1940) in which "perivascular round-cell infiltrations such as are described by most workers on swine fever are conspicuous by their absence."

In all, 46 cases of swine fever were encountered and the organisms isolated are listed in the table. In 30, no organism of known pathogenic importance was isolated, in four *Salmonella cholerae-suis* was found, in seven *Pasteurella suis* alone, in two *P. suis*, together with *Corynebacterium pyogenes*, and in a single case each *Alkaligenes* (*suis*), a staphylococcus and a "Group C" streptococcus.

MICRO-ORGANISMS ISOLATED FROM CASES OF DISEASE OTHER THAN SWINE FEVER (SEE TABLE)

Salmonella

S. cholerae-suis alone was obtained in six cases, together with *P. suis* in three and with *C. pyogenes* in one: *S. typhi-murium* alone was isolated from two.

In the six cases in which *S. cholerae-suis* alone was isolated, more than one organ was involved and *post-mortem* findings were typical for swine paratyphoid. In three cases *S. cholerae-suis* was isolated from more than one organ together with *P. suis* or *C. pyogenes* from the lungs; here again *post-mortem* findings were typical of swine paratyphoid. In one case *S. cholerae-suis* and *P. suis* were isolated from the lungs only; there was no ulceration of the intestines and the outstanding abnormalities were extensive pneumonia and pleurisy with adhesions.

S. typhi-murium was isolated twice. In one case in which it was obtained from spleen, liver and kidney, *post-mortem* examination

TABLE

PATHOGENIC ORGANISMS ISOLATED FROM SIXTEEN CASES OF SWINE FEVER AND TWENTY-SEVEN CASES OF DISEASE OTHER THAN SWINE FEVER

	S. cholerae-suis	*Pasteurella suis*	*Alkaligenes (suis)*	*S. typhimurium*	*Staphylococcus*	*Streptococcus Group "C"*
Swine fever ...	4	7	1		1	1
Disease other than swine fever ...	6	6	3	2		

	S. cholerae-suis + *Past. suis*	*S. cholerae-suis* + *C. pyogenes*	*Past. suis* + *C. pyogenes*	*Past. suis* + *Alk. (suis)*	*Past. suis* + *Staphylococcus*	*Alk. suis* + *C. pyogenes*
Swine fever ...			2			
Disease other than swine fever ...	3	1	2	2	1	1

revealed intestinal ulceration, peritonitis, pericarditis and pleuritis. In the other, in which it was obtained from lungs only, the animal had been dead for at least 24 hours and pleural adhesions constituted the only definite abnormality. *S. typhi-murium* is not generally recognised as a cause of swine paratyphoid, but according to Hutyra, Marek and Manninger (1938) it may be a rare secondary invader in cases of swine fever. The strains included in this paper were isolated from two pigs belonging to the same piggery and believed not to be affected with swine fever.

All the strains of *S. cholerae-suis* isolated were identical in biological characteristics and antigenic structure. They were agglutinated by antisera against whole group C, somatic antigen VI, somatic antigen VII, specific flagellar antigen c, and non-specific flagellar antigens 1, 5.

The two strains of *S. typhi-murium* isolated were identified serologically and agglutinated by the antisera against whole *typhi-murium*, somatic antigen IV, specific flagellar antigen i, and non-specific antigens 1, 2, 3.

Pasteurella

In six cases *Pasteurella* was isolated alone, in two cases with *C. pyogenes*, in three with *S. cholerae-suis*, in two with *Alkaligenes (suis)*, and in one with a β-haemolytic staphylococcus. Except in one case in which it was recovered from heart blood only, the *Pasteurella* was isolated from lungs alone, or from lungs and other organs.

In all cases in which *P. suis* was isolated some degree of pneumonia was present. In those cases in which *C. pyogenes* also was isolated, severe, purulent pneumonia and pleural adhesions were noted. Pneumonic lesions were also found in all three cases in which *S. cholerae-suis* was present, together with *P. suis*. The pathogenic importance of *P. suis* in Palestine is indicated by the fact that pneumonic lesions were always noted in cases from which it was isolated, and that it was not found in any case from the lungs of a series of 28 healthy pigs examined bacteriologically. No case of true Pasteurella septicaemia, as judged by *post-mortem* findings, was observed and in the three instances in which this organism was obtained from more than one organ in the same pig, visible macroscopic lesions were confined to the lungs.

In their main characteristics the 14 strains of Pasteurella isolated resembled closely the "typical" indol-producing, non-haemolytic haemorrhagic septicaemia organisms described by many authors (*e.g.*, Newsom and Cross, 1932). They were pathogenic for mice and on the basis of additional cultural and biochemical properties they come within the classification of porcine strains in Group II, as described by Rosenbusch and Merchant (1939).

Corynebacterium pyogenes

C. pyogenes occurred with *S. cholerae-suis* in one case, with *P. suis* in two and with *Alkaligenes* (*suis*) in one. It was isolated from the lungs alone which in all cases showed extensive, purulent pneumonia. The strains resembled closely those described by many authors (Magnusson, 1929; Merchant, 1935; Wilson and Miles, 1946) as being responsible for pathological conditions in animals.

Alkaligenes (*suis*)

This organism was isolated from the lungs only in six cases, three times alone, twice with *P. suis* and once with *C. pyogenes*. A patchy, white-marbled consolidation of the lungs was present in the three cases in which this organism alone was isolated, a moderate degree of pneumonia in the two in which it was present with *P. suis*, and extensive purulent pneumonia in the one in which *C. pyogenes* was also found. Spray (1922) has isolated it from the lungs of pigs, Thorp and Tanner (1940) found it in eleven out of 204 pig lungs examined, and Phillips (1942, 1944) regards it as pathogenic. It is believed to be a pathogen in Palestine, and in those cases in which it was isolated alone, the white-marbled consolidation of the lungs was unlike anything seen in other pneumonic conditions in pigs.

The strains were morphologically, culturally and biochemically indistinguishable from *Brucella bronchisepticus* as described by Bergey (1939).

Pathogenicity tests in mice were carried out with three strains, all of which proved lethal within 30 hours. They produced extensive

congestion of the internal organs and apparent enlargement of the small intestine. The organism was seen in large numbers in smears from lung, heart, liver, spleen and kidney, and was readily recovered in culture.

MISCELLANEOUS ORGANISMS AND ORGANISMS CONSIDERED TO BE *post-mortem* INVADERS

Streptococci

Miessner and Wetzel (1939), and Thorp and Tanner (1940) refer to the isolation of streptococci from diseased pigs, but in Palestine four strains only were recovered from the 100 pigs examined *post-mortem*. Two were diplococci, one of which did not ferment inulin and was non-pathogenic for mice. The second recovered from the lungs, liver, spleen and kidneys, was considered to be the cause of the death of a three-months-old pig. In some respects it resembled *Diplococcus pneumoniae* (Wilson and Miles, 1946) being capsulated, pathogenic for mice and able to ferment inulin ; it was, however, insoluble in bile. The third streptococcus was α-haemolytic, fermented inulin and was isolated from the liver of one case. The fourth, obtained in pure culture from the lungs, heart and kidney of a case of swine fever, was β-haemolytic, pathogenic for mice and similar to Group C, Sub-group 2 (Wilson and Miles, 1946).

Staphylococci

Staphylococci were recovered from the kidneys in one case of swine fever, and from the heart of one case of disease other than swine fever ; this latter strain was β-haemolytic but coagulase-negative.

Corynebacterium not *Pyogenes*

Corynebacteria which were not *C. pyogenes* were obtained from the lungs of one case and from the lungs, liver, spleen and kidney of another. In the latter case, it is probable that the pig, which was destroyed for *post-mortem* examination, was affected with foot-and-mouth disease, two outbreaks of which occurred among pigs during the course of this investigation.

Clostridium oedematiens

Cultures incubated under anaerobic conditions did not reveal the presence of *Clostridium oedematiens* which, according to Hutyra, Marek and Manninger (1938) may be an important secondary organism in some outbreaks of swine fever.

Post-mortem invaders

Many organisms were obtained from various cases—especially those in which putrefaction was advanced—in circumstances that

suggested that they were *post-mortem* invaders. Briefly, they can be described as organisms of the genera *Escherichia* and *Proteus*, organisms of the intermediate coli-aerogenes group and saprophytic spore-bearing anaerobes.

SUMMARY

Bacteriological findings at 100 *post-mortem* examinations of pigs (including 46 cases of swine fever) in Palestine, are recorded. The various organisms isolated are described and brief notes on *post-mortem* findings are given.

The organisms encountered which are generally accepted as of pathogenic importance, were *Salmonella cholerae-suis*, *S. typhi-murium*, *Pasteurella suis*, *Corynebacterium pyogenes* and *Alkaligenes (suis)*.

ACKNOWLEDGMENT

We wish to express our thanks to Miss G. Neuman, Senior Laboratory Assistant of this laboratory, for identifying most of the strains of Salmonella.

ADDENDUM

Shortly after this paper was written a case of swine fever was encountered in March, 1946, from which *S. dublin* was isolated from lungs and liver. It was agglutinated with antisera against IX, g, p, and mt: weak inter-agglutination of 1/50: (Kaufmann, 1939).

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THE BACTERIAL FLORA FOUND IN THE VAGINA OF DAIRY COWS WITH SPECIAL REFERENCE TO CORYNEBACTERIA

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INTRODUCTION

Studies on the bacterial flora of the vagina of normal or abnormal cows are not numerous.

Beaver (1922) made a survey of sterile cows and among the organisms he isolated were staphylococci, *B. subtilis*, non-haemolytic streptococci, *C. pyogenes* and green streptococci. He regarded the last two only as pathogenic. Attempts to infect two heifers with a culture of *C. pyogenes* failed. The same year Smith (1922) drew attention to a diphtheroid isolated from the urine of some normal calves which he thought might be of some significance. Jones and Little (1930) also recovered a similar organism from the urine of 12 out of 34 calves, mostly males, and in one case from the vagina of a healthy calf: they were satisfied that this diphtheroid was identical with *C. renale*. Symptoms resembling pyelonephritis followed the inoculation of this organism into cattle, although the disease was not reproduced by artificial infection with two strains recovered from natural cases of pyelonephritis. Sykora (1932) made a bacteriological survey of normal and sterile cows and concluded that most organisms isolated were saprophytes or secondary invaders. He made the point, however, that in the absence of lesions or clinical symptoms, abnormal cows may erroneously be regarded as normal. In addition, he reported that from the cervix of normal cows he isolated staphylococci in 75 per cent. of cases examined, the remaining 25 per cent. being sterile. He concluded that there is no qualitative difference in the flora of healthy and diseased cows (although *C. pyogenes* was isolated from diseased animals only) and that in sterile cows only an increase in numbers of organisms is apparent. Pascu (1939), who examined the flora of the vagina of normal cows noted that an increased number of coliforms was present around the urinary meatus and a higher degree of infection in the vestibule. In Japan, Ochi and Zaizen (1936) recovered *C. pyogenes* from samples of vaginal mucus in six out of eleven healthy cows and also from three samples of urine out of 32. The possibility of *C. pyogenes* occurring as a latent infective organism was stressed by Lovell (1939) when he concluded from a study of the normal antitoxin to *C. pyogenes* in animals that "there is evidence that the stimulus is a specific one and that the antitoxin is provided by the latent infection with *C. pyogenes*." He reported that Bosworth (Lovell, 1943) recovered *C. pyogenes* from the cervix of normal cattle. Smith and Sherman (1939) made a study of streptococci found in the vagina of normal cows. They recovered 27 strains of *Str. acidominimus*, but none of Group B streptococci (*Str. agalactiae*). Finally Francis (1941), in a bacterial survey of the vagina of 100 normal cows *post mortem* found *C. pyogenes*, *Str. dysgalactiae* (Group II streptococcus) and *Str. uberis* (Group III streptococcus) each once, and *Str. acidominimus* 15 times.

METHODS AND MATERIAL

Material from the normal animals was collected by means of a cotton wool swab introduced into the vagina to the level of the urinary meatus. Cultures were made by direct plating on blood agar plates and from the washings of the swab in 10 c.c. of sterile Ringer's solution containing 1 per cent. broth. In all cases the material was plated out on the day it was collected, as experience had shown that *C. pyogenes* does not survive long on cotton wool swabs. Plates were read after 48 hours' incubation and individual colonies picked for further investigation. Material which originated from diseased animals was sent by post to the laboratory and consisted of vaginal or uterine discharge. This was plated on blood agar and incubated for 48 hours.

Identification of organisms

The identification of coliform organisms, staphylococci and streptococci was made by the colonial appearance on blood agar and the microscopic examination of films stained by Gram's method. A representative number of streptococci was examined further by biochemical and serological methods but as none of the strains fell into well-defined species or serological groups they are described here by the type of haemolysis shown on blood agar plates. None of the strains of haemolytic staphylococci tested with rabbit plasma showed a positive coagulase reaction. Strains of *C. pyogenes* were identified by their morphology, haemolysis on blood agar plates, acidification and clotting of litmus milk and liquefaction of solid serum and gelatin.

The identification of *C. renale* was based chiefly on the findings of Jones and Little (1925, 1930) and Merchant (1935), which are summarised by Topley and Wilson (1946). A great deal of variation in the biochemical reactions of this organism is noted by these authors and others (Brooks, 1942; Thorp *et al.*, 1943; Feenstra *et al.*, 1945). All agree that this organism is sufficiently characterised by its morphology and typical diphtheroid characters, its inability to liquefy gelatin or solid serum, the absence of acid formation in litmus milk and the fermentation of glucose but not maltose, sucrose or lactose. Other sugars have sometimes been fermented by some strains. Of six strains isolated by the writer from naturally occurring cases of pyelonephritis in cattle, all fermented glucose, two also fermented sucrose and maltose, while none fermented lactose. All failed to produce acid in litmus milk, or to liquefy gelatin or solid serum. Any strain of diphtheroid isolated in this work which did not conform to these characters and which could not be classified as of another recognised species, was classed under "unidentified diphtheroids."*

* Since the completion of this work, Lovell (1946a) has described two further tests for the identification of *C. renale*, depending on the power of the organism to split urea and on the formation of a clear zone surrounding colonies of the organism grown on milk agar.

THE BACTERIAL FLORA OF NORMAL ANIMALS

A survey of three normal herds was made. One herd contained 26, another 20, and the third 54 cows, a total of 100. The results are shown in Table I.

TABLE I
BACTERIAL FLORA IN 100 NORMAL COWS

Organisms	*Herd* 1 (26 *cows*)		*Herd* 2 (20 *cows*)		*Herd* 3 (54 *cows*)		*Total* 100 *cows*
	Total	*Per-centage*	*Total*	*Per-percentage*	*Total*	*Per-centage*	
Green streptococci ...	23	95·8	20	100	53	98·1	96
Staphylococci (non-haem.)	15	57·6	12	60	25	46·2	52
C. renale	9	34·6	4	20	31	57·4	44
Bact. coli (non-haem.) ...	11	42·3	9	45	22	40·7	42
Unidentified diphtheroids	7	26·9	8	40	17	31·4	32
Staphylococci (haem.) ...	1	3·8	0	—	16	29·6	17
Bact. coli (haem.) ...	1	3·8	1	5	1	1·8	3
C. pyogenes	0	—	0	—	1	1·8	1

The only significant difference between the herds is the high incidence of haemolytic staphylococci and *C. renale* in herd 3. No explanation is available in regard to haemolytic staphylococci but it was known that several cases of pyelonephritis had occurred in this herd during recent years, a fact which may have some bearing on the increase in incidence of *C. renale* although all the cows which were examined were apparently normal. In view of the closeness of the findings in these three herds and similar observations over a number of other individual animals it seems probable that the picture presented in Table I may be taken as representative of the normal flora of the vagina. This view receives support from the findings in sterile cows from a large number of herds, in which no abnormal secretion or discharge could be detected. (See below.)

THE BACTERIAL FLORA IN STERILE COWS

For the present purpose, infertile animals were divided into those in which there is an abnormal secretion or discharge and those in which no such abnormality is present. In the latter the bacterial flora was similar to that of normal animals. The results of the examination of material from 180 animals with abnormal discharge and clinically classed as endometritis are shown in Table II.

It will be seen that, except for the absence of *C. renale*, the bacterial species present are similar to those isolated from normal animals (Table I). In some cases the incidence is broadly similar, *e.g.*, coliforms and haemolytic staphylococci. In others it is lower, such as green streptococci and non-haemolytic staphylococci, while "diphtheroids" (*C. pyogenes*,) were found only rarely in

discharges. These variations may be due to two factors. In the first place, a certain delay between the time of collection and the time of culturing the material from sterile cows, which might result in the death of certain organisms, was unavoidable ; secondly, the material consisted of discharge or secretion mainly originating from the uterus or the cervix and not from the vagina.

The results regarding *C. renale* suggest that this organism is present only in the posterior vagina, notably around the urinary meatus. As *C. renale* has been recovered many times from the urine of normal animals, the infection of the vagina may be due to urinary infection which had not spread to the anterior portion or cervix from which the material from sterile cows was collected.

The present study also demonstrates the presence of *C. renale* in the posterior urinary tract in approximately 40 per cent. or more of normal cows (Table I). This must be taken as a minimum figure as the results described here were all obtained from a single exam-

TABLE II

BACTERIAL FLORA OF DISCHARGES FROM 180 INFERTILE COWS

Organism	*Number of times isolated*	*Per cent. of total*
B. coli (non-haem.)	78	42·7
Green streptococci	63	35·0
C. pyogenes	50	27·6
Staphylococci (haem.)	48	26·6
Staphylococci (non-haem.) ...	14	7·7
B. coli (haem.)	12	6·6
Anthracoids	10	5·6
Diphtheroids (not *C. pyogenes*) ...	2	1·1

ination, whereas repeated examination of an individual animal can reveal the presence of the organism when a single examination has failed to do so. In the past, the source of infection in cases of pyelonephritis of cattle has been discussed as being of haematogenous or urinary origin. Lovell (1946b) showed that mice can be infected with *C. renale* by the haematogenous route, but failed to infect them *per vaginam*. The present study indicates that there is no necessity to postulate a haematogenous infection in cows since certain factors which are not understood at present might allow the organism normally present in the urinary tract or vagina to become pathogenic ; nor would there be any need for transmission of infection from cow to cow as it is likely that many or most animals are potentially infected.

The most striking result, however, is the isolation of *C. pyogenes* in 27·6 per cent. of the copious purulent discharges in contrast with its rare occurrence in normal cows, or in sterile cows without abnormal discharge. When found, it was usually present in very large numbers and frequently in pure culture.

C. pyogenes was not found to be a normal inhabitant of the vagina but is evidently of importance in certain well-defined cases of sterility in cows and is associated with a characteristic discharge. It seems likely that the bacteria in other discharges, which are commonly of a mucoid nature, are derived from the normal flora of the vagina.

No reference is made to *Br. abortus* and *M. tuberculosis* in the course of this study.

SUMMARY

A survey of the flora of the vagina of 100 apparently normal dairy cows is reported.

C. renale is a frequent and possibly normal inhabitant of the posterior urinary tract of dairy cows. Its significance is discussed.

C. pyogenes is associated with an endometritis in cows characterised by copious purulent discharge.

Apart from *C. pyogenes* there is no evidence of other organisms being associated with sterility in cows.

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TYPES OF CELLS PRESENT IN COW'S MILK

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INTRODUCTION

The present study was undertaken in order to establish the types and origin of cells present in milk from normal cows throughout a lactation period and of cells present in milk from cows suffering from a more or less obvious mastitis. These fundamental points are at present a matter of controversy, as can readily be seen from the literature on this subject.

As far as normal milk is concerned, three divergent viewpoints can be distinguished. The first (Winkler, 1908; Hewlett *et al.*, 1909) states that epithelial cells from the mammary gland form the main part of cellular structures of normal milk and that there is no real evidence that leucocytes are present. The second group (Kirksteins, 1917; Bourgeois, 1927; Christiansen, 1929; Sauferlein, 1930; Tapernoux, 1931; Bachmann, 1932; Grassi, 1932; Holm, 1934; Duran-Jorda, 1944) regards the leucocytes as the main cellular elements of milk, and does not pay very much attention to the epithelial cells. In this group the views of Holm and of Sauferlein are rather different from others, as Holm believes that only lymphocytes, both large and small, are present in normal milk and Sauferlein found only macrophages. The third school (Breed, 1914; Varrier-Jones, 1924) seems to compromise between the two former groups, considering both epithelial cells and leucocytes as equal partners in constituting the cellular structures of normal milk.

As regards milk from cows affected with mastitis, the differences of opinion expressed by various writers are also very great. Thus, Winkler (1908) and Hewlett *et al.* (1910) consider the majority of the cells in mastitic milk to be epithelial; they believe also that the multinucleated cells which may resemble in certain respects polymorphonuclear leucocytes, are in fact epithelial in origin. Another school of thought (Meinert and Weigmann, 1909; Lewis, 1914; Varrier-Jones, 1924; Sauferlein, 1930; Holm, 1934) believes that polymorphonuclear neutrophil leucocytes are either absent from normal milk or present in very negligible numbers and has stated that the appearance of fair numbers of these cells in milk is indicative of mastitis. Bartsch (1934), on the other hand, is of the opinion that eosinophils in the milk should be regarded as an indication of mastitis. He believes that they are formed in the udder and that they amount to 0·3 per cent. of the total number of cells in normal milk. In cases of retention of milk he found that they increased up to 5 per cent. and in acute streptococcal mastitis their numbers increase

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very considerably. Finally, there is a large group (Rautmann, 1928; Niemeyer, 1929; Wirth, 1929; Schönberg, 1930; Tapernoux, 1931; Bachmann, 1932; Pröscholdt, 1933; Schumann and Lorenzen, 1933), who observed the presence of both polymorphonuclear neutrophil leucocytes and lymphocytes in normal milk and who claim that in mastitis there is a great increase in the number of neutrophils as well as increases in numbers of other leucocytes and epithelial cells.

It is clear from this short summary of previous work on the subject that a clarification on the lines mentioned in the introductory sentence is necessary.

MATERIAL AND METHODS

The material used in the present investigation consisted of fore-milk samples, drawn from individual quarters of seven heifers throughout a whole lactation period; for the first five weeks samples were taken every second day, but later at weekly intervals only. At the beginning of the lactation period all the cows were apparently healthy and did not exhibit any abnormality of their udders. The animals were rather poor milkers and during the short course of lactation none of them became pregnant, so that the findings are of a single, short, first and uncomplicated lactation in poor milking cows. Some of the cows were subjected to various experiments during the course of lactation in order to induce mastitis (Dr. McEwen).

From a well-shaken large sample of fore-milk 5 ml. were centrifuged at about 3,000 revolutions per minute for 15 minutes. The cream and supernatant fluid were carefully removed and the deposit was spread on a slide. The film was then air-dried, stained at once with Leishman's stain and later a differential cell count was made, using 2 mm. oil-immersion objective and counting 100 cells. It is worth noting that samples of cream from the centrifuge tubes were occasionally examined; the number of cells present proved to be negligible.

CELL TYPES OF NORMAL MILK

All the types of cells present in milk samples obtained from normal cows are also found, with certain new types of cells, in samples of milk from cows suffering from a more or less obvious mastitis. For this reason it is satisfactory to give a systematic description of all the types of cells present in the bovine fore-milk and later to discuss the proportions of the various cell types in health and disease.

Epithelial cells.

These cells are derived from the epithelial lining of acini and ducts of the mammary gland. In an attempt to differentiate between the two types (acini and ducts), smears were made from various parts of the mammary gland—cistern, large ducts and

parenchyme. This effort proved entirely unsuccessful, as the cells from any particular region vary greatly in shape and in size and the different regions show similar variations. The epithelial cells in the milk can be divided into six main groups. This differentiation, however, is not an absolute one, as several intermediate forms may be observed.

(*a*) *Epithelial cells with clear cytoplasm* (size 9–15μ).—The nucleus of these cells is usually well defined, spherical or elliptical in shape. The amount of clear cytoplasm around the nucleus varies from a very narrow, hardly visible zone (Fig. 7a) to a comparatively broad zone with slightly darker borders (Fig. 1). These cells are completely devoid of fat vacuoles.

(*b*) *Epithelial cells with neutrophilic foamy cytoplasm* (size 12–17μ).—These cells contain a spherical or elliptical nucleus, but in many cases fat vacuoles are seen within the nuclear substance and are associated with distortion of the nucleus (Figs. 4, 22a). The cytoplasm, which varies in amount, is colourless or just bluish and is loaded with fat vacuoles of various sizes. In cells where fat vacuoles have made their appearance in the nuclear matter, small dark purple granules are visible inside the cytoplasm. These granules probably are of nuclear origin, as their appearance and staining properties are similar to those of the nucleus (Fig. 4).

(*c*) *Epithelial cells with acidophilic foamy cytoplasm* (size 11–17μ).—The nucleus of these cells is spherical or irregular in outline. The cytoplasm stains either pink or red and appears to be filled with fat vacuoles. Generally the cells are very similar to those of the previous group, except that the cytoplasm is acidophilic. The shape of the nucleus, which is very often distorted, suggests, however, that these cells are in an advanced state of degeneration (Figs. 2a, 2b).

(*d*) *Epithelial cells with basophilic foamy cytoplasm* (size 10–25μ).—These cells are very similar to those with acidophilic cytoplasm, but the latter stains either a distinct blue or a very dark-blue colour. The cells with the blue cytoplasm have a large nucleus which shows very frequently different degrees of distortion and is often penetrated by fat vacuoles. The cytoplasm varies in amount and is completely packed with fat vacuoles (Fig. 3). The cells with the very dark-blue cytoplasm are usually smaller in size, the nucleus being small, very dark, pyknotic and very irregular in shape. The cytoplasm is rather granular in appearance and contains only a few fat vacuoles of a very large size (Fig. 5). In certain cells of the latter type the nucleus, through a lytic process, completely disappears leaving only cytoplasm filled with fat vacuoles (Fig. 11a).

(*e*) *Degenerated epithelial cells with polymorphic nuclei* (*pseudo-polymorphs*) (size 6–20μ).—These cells, which have frequently in the past been called neutrophil leucocytes, according to my observations originate from epithelial cells and exhibit a great variety of transitional stages from typical mononuclear epithelial cells to cells

very similar in appearance to neutrophil polymorphonuclear leucocytes. The pseudopolymorphs, which vary in shape and in size, have a cytoplasm which in the early stages of their development may be filled with fat vacuoles, but in the final stages, apart from a few lightly coloured pink granules, is completely clear and devoid of fat.

The development of the pseudopolymorphs was studied very carefully and various stages are illustrated in this paper in order to prove their epithelial origin. It has been ascertained that, in the majority of cases, the first change associated with the division of the nuclei of the epithelial cells was the presence of fat vacuoles within the substance of the nucleus. Fragmentation, pyknosis or karyorrhexis also occurs since cells devoid of fat showing early stages of division of the nuclei were observed.

The results of intra and extra nuclear pressure of fat is that the nucleus becomes reduced to a narrow sickle-shaped mass situated at one side of the cell (Fig. 6), is divided into two irregularly shaped parts which may be completely separated (Figs. 8b, 11b) or connected by thin strands of nucleoplasm (Figs. 10a, 10b, 13a), or in certain cells may become divided into three and more fragments (Figs. 9, 12). All the stages mentioned above are very distinctly different from neutrophil polymorphonuclear leucocytes and no mistake should be made when carrying out a differential count ; the danger of confusion is, however, very great when further stages of division of the nuclei of the pseudopolymorphs are encountered. These final stages provide a variety of cells which vary in size from 8μ to 16μ and have very distinctly pyknotic nuclei. The nucleus may take the form of an elongate mass connected by strands with small particles of nucleoplasm (Fig. 13b), it may consist of a shrunken body with one or more processes (Fig. 14), or lastly it looks very much like that of a neutrophil polymorphonuclear leucocyte being composed of a number of fragments, usually very darkly stained and homogeneous in appearance, either joined together by thin fibres of nucleoplasm (Fig. 15) or completely separated (Fig. 16).

Another type of pseudopolymorph which is quite different from that previously described is the basophilic pseudopolymorph. This is a perfectly spherical cell of small size, about $6–7\mu$ in diameter, with a very small pyknotic polymorph nucleus and a dark-blue foamy cytoplasm (Fig. 17). The origin of this cell was traced back to the epithelial cell with very dark-blue basophilic cytoplasm (Fig. 5).

(*f*) *Micro-pseudocells* (size 4μ).—These are very small structures which contain a small pyknotic nucleus and a small amount of cytoplasm usually attached to one side of the nucleus (Fig. 18). These cells are derived from the final degenerative stages of the pseudopolymorphs ; as has been pointed out, the nucleus of the latter cells often breaks up and each particle, with a small amount of cytoplasm attached to it, forms a separate unit (Fig. 16).

Neutrophil polymorphonuclear leucocytes.—These cells, when found in milk, do not differ from those observed in smears of bovine blood. They vary from 9–12μ in diameter and are round, elliptical or irregular in outline. The cytoplasm is very clear and usually contains a few lightly coloured pink granules. The nucleus stains very deeply and is formed of a thick chromatin network. Three to five lobes are seen most commonly and are often held together by very fine threads of nucleoplasm (Figs. 20a, 20b). Apart from these typical leucocytes, cells are observed which are very difficult to classify as they resemble both true leucocytes and pseudopolymorphs. They are larger in size than the typical leucocytes, about 15–16μ in diameter, spherical in shape and the cytoplasm is either clear, or contains fine pink granulations. The nucleus, as a rule, exhibits a coarse chromatin network and is either very large and irregular in shape or is composed of four to five lobes arranged in the form of a rosette with short, thin chromatin fibres which meet in the centre of the cell (Fig. 19). These cells are classed as atypical forms of leucocytes in cases when the cell picture reveals several true neutrophils ; on the other hand, when the majority of cells in a slide are epithelial and pseudopolymorphs of epithelial origin are common, these atypical cells are classed as pseudopolymorphs.

Coarsely granular eosinophils.—A large variety of cells which contain coarse eosinophilic granules was observed in some of the milk samples. The size of these cells ranges from 12–16μ in diameter ; they are either round or irregular in outline, their cytoplasm is clear and is filled with dark red, round granules. The number of granules differs from cell to cell ; in some of the cells the number is very large and they fill the entire cytoplasm ; in other cells, however, the number is much smaller and they are heaped at one side of the nucleus. The latter is variable in shape and may be spherical (Fig. 21), elongate (Fig. 22b), composed of two lobes (Figs. 23), or of three and more lobes (Fig. 24). These cells, although differing in size, shape of nucleus and number of eosinophil granules from eosinophil leucocytes present in smears of bovine blood, are considered to be true acidophil leucocytes ; the morphological differences in these cells are attributed to a degenerative process.

Lymphocytes.—In bovine milk the lymphocytes are spherical cells 6–9μ in diameter. The nucleus is very dark in colour, is composed of a very thick chromatin network and contains a marked indentation. In the small lymphocytes the amount of cytoplasm is very small and sometimes it is visible only at the indentation of the nucleus (Figs. 26a, 26b). In the larger lymphocytes the layer of cytoplasm surrounding the spherical nucleus varies from a narrow to a broad zone (Fig. 27a). Some of the nuclei have a very deep indentation so that they appear constricted (Fig. 27b) ; other cells, on the other hand, may contain a nucleus divided into two parts (Fig. 28).

Monocytes.—Monocytes, similar to those present in the blood, are occasionally found in milk smears. These are round cells, measuring 12μ and containing a clear blue cytoplasm with a few azurophil granules. The nucleus is composed of a thick chromatin network, is large in size, eccentric in position and is oval, kidney-shaped or deeply constricted (Fig. 25).

Large squamous cells (derived either from the streak canal or from the skin of the teat).—These are the largest cells met with in milk smears ; their proportion is very low in milk from both healthy and mastitic cows. They are usually very irregular in shape and measure 30–55μ. The cytoplasm stains a greenish colour with Leishman's stain and is granular in appearance. Within the cytoplasm varying numbers of micro-organisms are present which tend to accumulate at one part of the cell. The nucleus is very dark or exhibits a granular network of chromatin and is either spherical or more often elliptical in shape. The size of the nucleus, when compared with that of the cytoplasm, is very small as it measures 6–7μ only.

Red blood corpuscles.—Only rarely are these cells found in milk samples, but when present, they are always slightly deformed and are usually collected in small groups.

Debris of epithelial cells and free nuclei.—In all milk samples varying amounts of cell debris and free nuclei are observed. They represent a variety of structures from partly broken-up epithelial cells or nuclei to small fragments or completely deformed cells which cannot be classed at all.

TABLE I

PROPORTIONS OF THE VARIOUS CELL TYPES OF NORMAL MILK

Type of cell	*Average variation*	*Highest count*	*Lowest count*
Epithelial cells	55–85 per cent.	100 per cent.	14 per cent.
Pseudopolymorphs ...	6–40 „	72 „	0
Micro-pseudocells ...	1–20 „	43 „	0
Neutrophils	0	3 „	0
Eosinophils	0	0	0
Lymphocytes	1–18 „	30 „	0
Monocytes	0	0	0
Large squamous cells ...	1–5 „	28 „	0

The results of the present investigation prove beyond doubt that the greatest proportion of cells present in milk from normal cows is derived from the epithelial lining of the mammary gland. Careful consideration was given to the study of epithelial cells ; the morphology of the epithelial cells present in milk was compared over and over again with that of epithelial cells obtained from smears made of various parts of the mammary gland of cows and also with cells observed in histological preparations.

Epithelial cells of the six varieties, and occasionally large squamous cells, form the main bulk of cellular structures of normal milk throughout the lactation period. During the first three to four weeks of the lactation period, however, a fair number (1–18 per cent.) of small and large lymphocytes is quite common : later, lymphocytes are much less common and are usually found only in small numbers (1–3 per cent.).

The proportion of epithelial cells in relation to that of epithelial pseudopolymorphs and micro-pseudocells seems to vary from day to day. Fluctuations are very common, but it can be said that the number of epithelial cells (groups a, b, c, d) seldom falls below 40 per cent. and usually ranges between 55–85 per cent. ; the number of pseudopolymorphs, on the other hand, seldom rises above 60 per cent. and as a rule keeps between 6–40 per cent. ; finally, the incidence of micro-pseudocells is 1–20 per cent. and as a rule they appear to be fewer in number at the beginning of the lactation period. The number of squamous cells is constantly very small, they are only rarely observed, but when present they form 1–5 per cent. of the total number of cells.

True polymorphonuclear leucocytes of both the neutrophil and eosinophil varieties, monocytes and red blood corpuscles are almost invariably absent from normal milk. In a few instances neutrophils were observed in milk obtained from normal cows ; in these cases, however, they were very few in number (1–3 per cent.), they were never typical, rather similar to pseudopolymorphs and usually appeared at the beginning of the lactation period.

CELL TYPES OF ABNORMAL MILK

As already mentioned, epithelial cells, pseudopolymorphs, micro-pseudocells, squamous cells and lymphocytes, all in varying proportions, appear in milk from mastitis cows. As in normal milk, the ratio of these cells fluctuates daily and it was not possible to base any conclusions on the number of those cells. Apart from the already enumerated cells, leucocytes of the polymorphonuclear neutrophil and eosinophil varieties, monocytes and occasionally red blood corpuscles are common in milk from cows suffering from mastitis.

Out of the seven cows from which samples of milk were examined, five cows showed bacteriological evidence of infection with *Streptococcus agalactiae* in 13 quarters, one cow developed a clinical staphylococcal mastitis in the left hindquarter while the seventh cow proved to be bacteriologically free of infection.

Of the five cows infected with *Str. agalactiae*, only one cow showed a consistent high number of eosinophils in the milk from all four quarters, the count on certain days being 27 per cent. of the total number of cells present. In the remaining cows eosinophils were also found, but their number ranged from 0–5 per cent.

Polymorphonuclear neutrophil leucocytes were the most common abnormal cellular constituents of milk in streptococcal mastitis and in certain cases they formed 28 per cent. of the total cell count. One of the most important observations made in the course of the present investigation was the fact that neutrophils appeared in the milk in varying numbers, even up to 22 per cent., at the beginning of the mastitic process at the time when no other signs of the disease could be detected, and the total cell count was as low as 2,500 cells per ml. ; it was only after a lapse of days, or even weeks, that the same cow developed a high cell count.

Table II

Proportions of the Various Cell Types of Abnormal Milk

Type of cell	*Average variation*	*Highest count*	*Lowest count*
Epithelial cells	55–85 per cent.	98 per cent.	18 per cent.
Pseudopolymorphs ...	6–40 „	58 „	2 „
Micro-pseudocells ...	1–10 „	21 „	0
Neutrophils	1–25 „	28 „	0
Eosinophils	1–5 „	27 „	0
Lymphocytes	1–3 „	30 „	0
Monocytes	0–3 „	5 „	0
Large squamous cells ...	1–3 „	4 „	0

Monocytes in small numbers, up to 3 per cent., and red blood corpuscles in varying numbers, may form a part of the abnormal constituents of the cellular elements of milk. As far as monocytes are concerned, I have never observed their presence in samples of normal milk and I believe that in each case their appearance is an indication of an abnormal state of the udder.

The cow which developed staphylococcal mastitis supplies evidence that in this form of disease the neutrophil test is very sensitive. The cow calved on July 7th, and from July 8th until 19th there were no neutrophils present in the milk. On July 23rd, 2 per cent neutrophils were counted out of a total 2,000 cells per ml. of milk from the left hindquarter ; on July 25th, the number of neutrophils in the milk from the same quarter rose to 10 per cent. out of a total of 1,500 cells per ml. On July 27th the number of neutrophils fell to 1 per cent. of a total 1,500 cells per ml. From July 30th till August 10th there were no neutrophils in the milk from that quarter and the total cell count remained low, about 1,000 cells per ml. On August 13th, 2 per cent. neutrophils appeared in the milk of the left hindquarter and the total cell count rose to 2,030,000 cells per ml. ; on August 20th, the number of neutrophils was 11 per cent. of the total cell count and the cow exhibited all the symptoms of a clinical staphylococcal mastitis.

DISCUSSION

The results of the present investigation prove that a large proportion of cellular elements, present in either normal or abnormal milk, consists of epithelial cells of the mammary gland ; in view of this fact the commonly used term " leucocyte count " should be replaced by the more correct " cell count."

Winkler (1908) and Hewlett *et al.* (1909) are of the opinion that normal milk contains epithelial cells mainly and in no case have they observed true neutrophils in the milk ; multinucleated epithelial cells, according to these authors, appear in various proportions in normal milk throughout the lactation period. These views are in contrast to those of Varrier-Jones (1924), who found a small proportion of epithelial cells only in normal milk, the majority of cell elements being lymphocytes. As to the multinucleated epithelial cells, Varrier-Jones believes that they are tissue cells and not leucocytes ; he further believes that they appear in normal milk at the end of the lactation period only as so-called " drying-off cells." My own results are rather similar to those of Winkler and Hewlett, *et al.*, as the majority of cells which I have observed in normal milk were epithelial cells, both uni- and multinucleated, the latter being present throughout the lactation period. As far as the views of Sauferlein (1930) and of Holm (1934) are concerned, I am in complete agreement that polymorph neutrophils are not present in normal milk, but at the same time I believe that the macrophages filled with fat of Sauferlein are in fact epithelial cells, and whilst some of Holm's lymphocytes are undoubtedly true lymphocytes : others, possibly the majority, are epithelial cells. It is surprising that these workers did not observe multinucleated cells in normal milk, as in the present investigation the number of pseudopolymorphs was in certain cases as high as 60 per cent. of the total number of cells.

There is little agreement as to the occurrence of true polymorphonuclear neutrophil leucocytes in normal milk. Thus, Winkler (1908), Hewlett *et al.* (1909, 1910), Sauferlein (1930) and Holm (1934) deny their presence in normal milk. Varrier-Jones (1924) and the present writer believe that occasionally a negligible proportion of these cells may be observed in milk which, as far as can be determined, is normal. Breed (1914) draws attention to the fact that, although a large proportion of cellular elements of milk are epithelial cells, neutrophils are also present in fair numbers. The latter originate from the blood stream, and according to Breed make their way into the milk by passage through the epithelial lining of the mammary gland. Similar observations to those of Breed were made by Emmel *et al.* (1926), who noticed that during active nursing an increased passage of lymphoid cells into the alveoli and of neutrophils into the ducts takes place simultaneously with a decrease of the same elements in the circulating blood. Other writers (Kirksteins, 1917 ; Bourgeois, 1927 ; Christiansen, 1929 ; Tapernoux, 1931 ; Bachmann, 1932 ; Grassi, 1932) divide the cells found in milk into groups

similar to those arranged for the blood cells and some claim that neutrophils form a large proportion of the cell elements of normal milk (Bachmann, 41·3–67·4 per cent. ; Grassi, 48 per cent.). It seems obvious that by so doing this last group of workers (Kirksteins, etc.) have disregarded the normal physiology of the mammary gland. If we assume that the results of Emmel *et al.* are correct in that the majority of blood cells which pass into the milk are lymphoid in character, the figures given by Bachmann and Grassi for the neutrophils appear extremely high. In my opinion, the majority of these neutrophils are in fact pseudopolymorphs, that is epithelial cells apparently multinucleated as a result of the changes associated with the various stages of nuclear degeneration.

Duran-Jorda (1944) states that milk-fat is secreted by a " kind of polymorph neutrophil " which carries fat to the mammary gland, discharges its fat content into the acini and later changes its morphology into a cell similar in appearance to a lymphocyte. This statement is in complete opposition to the observations, not only of the workers on the cellular elements of normal milk but also to all the recent research on the histology, cytology and physiology of the mammary gland. It is quite true that some research workers believe that leucocytes, particularly lymphocytes, take part in the secretion of milk, but merely by adding their bodies to the secretion of the mammary gland, the latter being solely responsible for the production and secretion of fat. The correlation between uni- and multinuclear cells in the milk, observed by Duran-Jorda, has also been recorded in the present paper. My observations, which diametrically oppose the observations of Duran-Jorda, show that uninuclear epithelial cells of the mammary acini and ducts become shed off and extruded into the acini and ducts where they may undergo degeneration with a fragmentation of their nuclei and give rise to the formation of pseudopolymorphs. The photomicrographs shown by Duran-Jorda suggest that his mononuclear cells are just epithelial cells, while the " neutrophil polymorphs " are very similar, if not identical, with the pseudopolymorphs described in the present paper.

Referring to the cell content of milk from cows suffering from mastitis, Winkler (1908) and Hewlett *et al.* (1910) claim that in no instance have they observed true neutrophil leucocytes in milk deposits and they state that the only cells which could be mistaken for neutrophils are multinucleated epithelial cells. These writers conclude that in their opinion there is no marked difference in the cell picture of normal and mastitic milk. The results of the present investigation which differ from those of the writers mentioned above, are rather similar to those made by Meinert and Weigmann (1909), Lewis (1914), Varrier-Jones (1924), Sauferlein (1930), and Holm (1934). In the majority of cases a clear distinction can be made between neutrophil leucocytes and multinucleated epithelial cells (pseudopolymorphs) ; further, I believe that any appearance of true

neutrophils above 2–3 per cent. of the total number of cells definitely indicates an abnormal state of the mammary gland. There are, however, good grounds for the belief that any presence of neutrophils is abnormal. These facts have been previously emphasised by Varrier-Jones, who draws attention to the similarity between the multinucleated epithelial cells and true neutrophils. He also states that a small proportion of true neutrophils (finely granular eosinophile cell) is not indicative of mastitis and advises in each case a differential count. Sauferlein, on the other hand, although making distinction between neutrophil leucocytes and multinucleated pseudopolymorphs, regards the latter as partly devoured epithelial cells and believes that these cells appear in mastitis only. The present results are in agreement with Holm's statement as regards true neutrophils, but I should like to draw attention to the fact that as Holm does not differentiate between true neutrophils and pseudopolymorphs, his conclusions may be erroneous.

Eosinophils, in negligible proportions, were occassionally found in the course of the present investigation in milk from mastitis cows and in the four quarters of one only out of the seven cows the number of eosinophils reached a figure as high as 27 per cent. of the total cell count. I cannot agree, therefore, with Bartsch (1934) that in each case of milk retention and mastitis the number of eosinophils increases considerably.

It is very difficult to comment on the results obtained by those workers who record very high figures for neutrophils in normal milk and substantial increases in milk from mastitis cows, but as they did not make allowance for epithelial pseudopolymorphs, the possibility of confusing the two types of cells can not be dismissed.

Finally, I should like to add that my observations are in agreement with Grassi's statement that leucocyte invasion begins at the onset of mastitis, even before it is clinically manifest.

SUMMARY

The various types of cells present in the milk are described.

In both normal and abnormal milk epithelial cells form a large proportion of the cellular elements.

Multi-nucleated epithelial cells (pseudopolymorphs) and micro-pseudocells originate from the epithelial lining of the mammary gland.

Apart from lymphocytes of both large and small varieties, no other leucocytes are usually found in normal milk.

Neutrophil polymorphonuclear leucocytes exhibiting atypical morphology, may be present in milk presumed normal in very small numbers, usually not exceeding 2–3 per cent. of the total number of cells.

The appearance of typical neutrophils and eosinophils in milk deposits obtained from apparently healthy cows, should be looked upon as an early indication of a developing mastitis.

Neutrophils are by far the most common abnormal cellular elements of milk, but in some cases the number of eosinophils may exceed that of the neutrophils.

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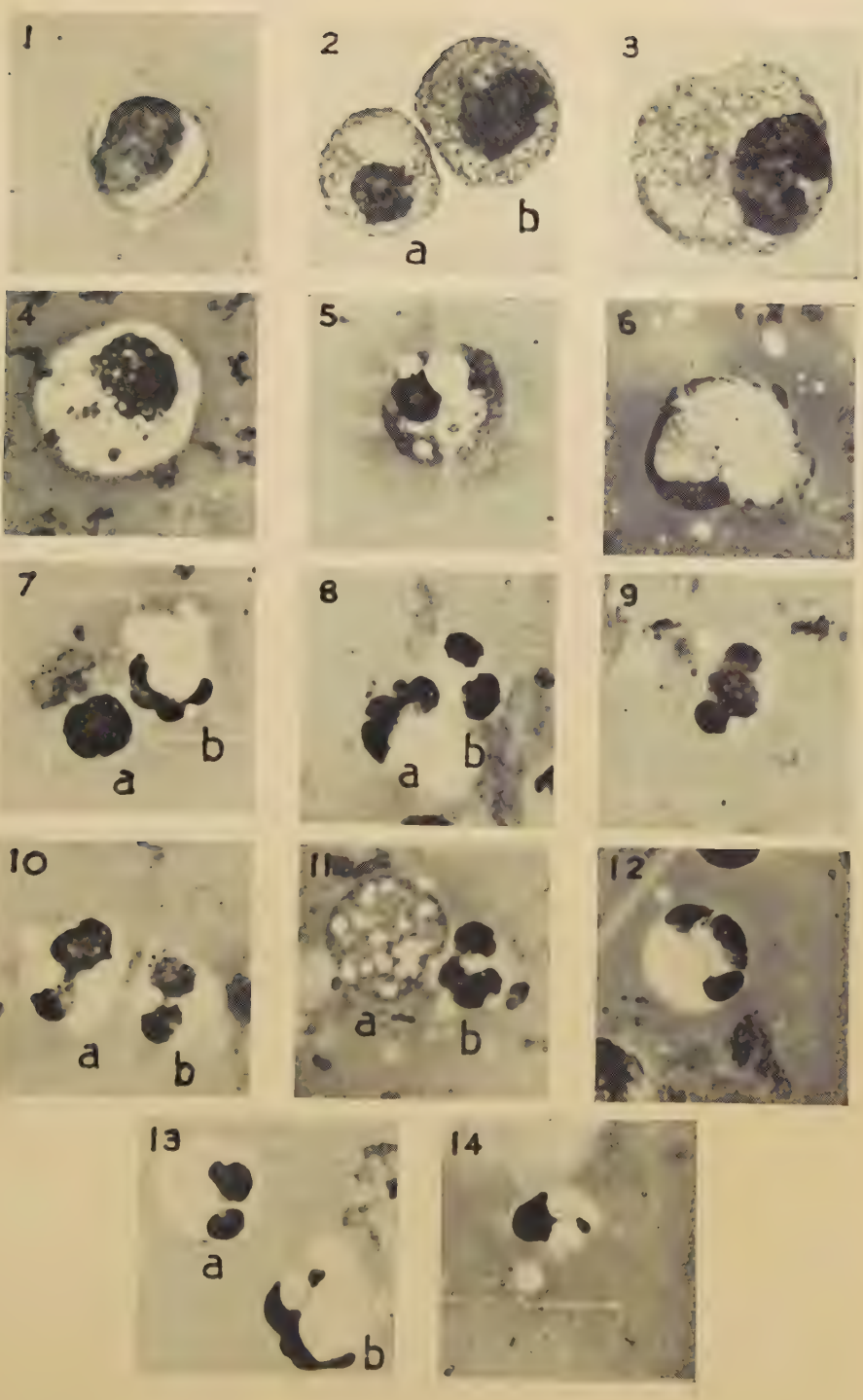
All illustrations consist of unretouched photomicrographs, taken at a magnification of 1100.

FIG. 1.—Epithelial cell with clear cytoplasm.
FIG. 2 (*a* and *b*).—Epithelial cells with acidophilic foamy cytoplasm.
FIG. 3.—Epithelial cells with basophilic foamy cytoplasm.
FIG. 4.—Epithelial cell with neutrophilic foamy cytoplasm.
FIG. 5.—Epithelial cell with very dark blue foamy cytoplasm.
FIGS. 6, 7*b*, 8*a*, 8*b*—Pseudopolymorphs.
FIG. 7*a*—Epithelial cell with clear very scanty cytoplasm.
FIGS. 9, 10, 11*b*, 12, 13, 14.—Various stages of pseudopolymorphs.
FIG. 11*a*.—Epithelial cell with dark blue foamy cytoplasm completely devoid of nuclei.
FIGS. 15, 16.—Pseudopolymorphs.
FIG. 17.—Basophilic pseudopolymorph.
FIG. 18.—Micro-pseudocell.
FIG. 19.—Atypical neutrophils.
FIGS. 20*a*, 20*b*—Neutrophils.
FIGS. 21, 22*b*, 23, 24.—Eosinophils.
FIG. 22*a*.—Epithelial cell with neutrophilic foamy cytoplasm.
FIG. 25.—Monocyte.
FIGS. 26*a*, 27*a*.—Large lymphocytes.
FIGS. 26*b*, 26*c*.—Small lymphocytes.
FIG. 27*b*.—Large lymphocyte with a constricted nucleus.
FIG. 28.—Large lymphocyte with a dividing nucleus.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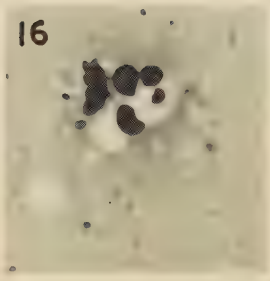
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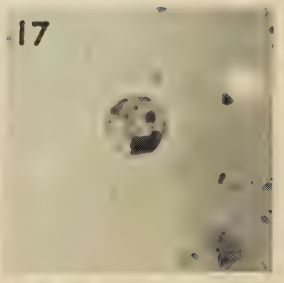
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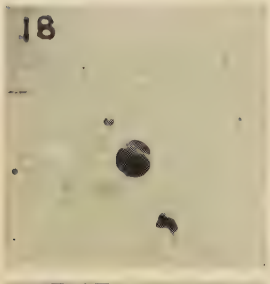
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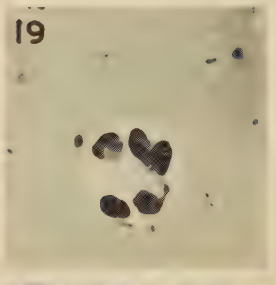
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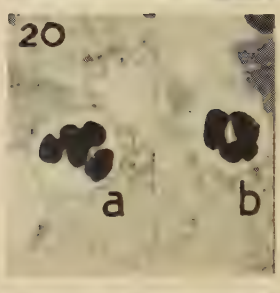
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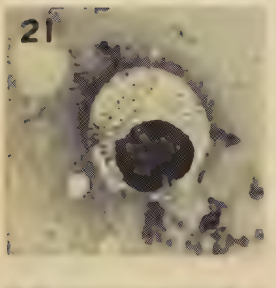
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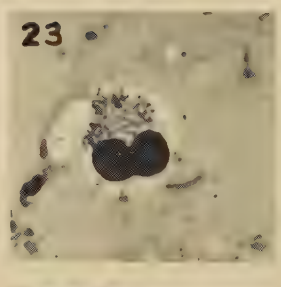
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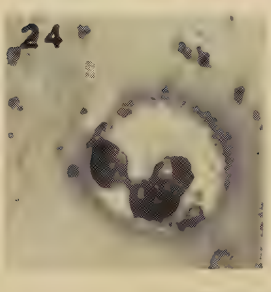
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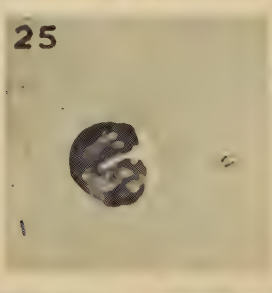
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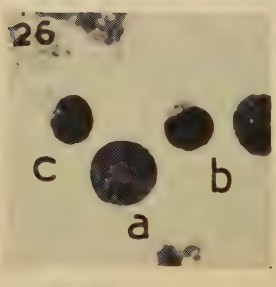
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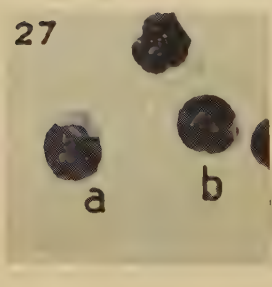
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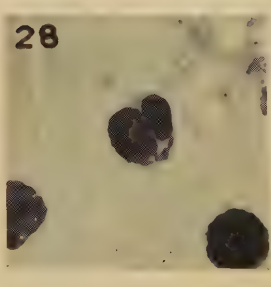
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SULPHONAMIDE INVESTIGATIONS

II. Treatment of Experimental *Pasteurella septica*, *Ery. rhusiopathiae* and *S. cholerae-suis* Infections in Mice with Sulphathiazole, Sulphadiazine, Sulphamezathine and Sulphapyridine

By

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INTRODUCTION

These experiments in mice formed part of a study of the sensitivity of the common animal pathogens to various sulphonamide drugs. *In vitro* experiments have also been made on a wider range of organisms and will be reported separately. This work formed part of the general investigation on the veterinary applications of sulphonamide drugs referred to in the first paper of this series (Maclay and Slavin, 1947).

The present report deals with the sensitivity of *P. septica* (avian and bovine strains), *Ery. rhusiopathiae*, a Group C Streptococcus, and *S. cholerae suis* to sulphathiazole, sulphadiazine, sulphamezathine and sulphapyridine as compared with sulphanilamide.

METHODS

The mice used were females of the Compton strain of white mice (average weight 18 grammes), and were fed on a diet of oats and bread soaked with water. They were used in groups of ten or 20 animals, one or more groups being left as untreated controls.

They were infected by intraperitoneal inoculation of suitably diluted cultures, a preliminary titration of the virulence of each organism in mice being first made and an inoculum selected varying from ten times to over 1,000 times the $L.D._{50}$. In all experiments counts of the numbers of viable organisms inoculated were made by pour-plates.

In the earlier work with pasteurella, dilutions of culture for both the preliminary titration and the treatment experiments were made in saline (Experiments 1 and 2) or Ringer's solution (Experiment 3), and no difficulty was encountered. When *Ery. rhusiopathiae* came to be tested, however, it was found that Ringer's fluid was unsatisfactory in that although a number of organisms was inoculated, which proved viable by plate counts and which was judged from preliminary tests to be well above the lethal dose, the control mice failed to die. The preliminary virulence titrations in this case had been done earlier using broth as the diluting fluid. It appeared

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that in Ringer's solution the virulence of *Ery. rhusiopathiae* for mice was rapidly destroyed, while the organism itself died more slowly.*

We wished to avoid, if possible, injecting into the mice the relatively large amount of peptone contained in broth, knowing that it was a sulphonamide antagonist, and various alternative diluting fluids were therefore tried. Finally, it was found that the virulence of *Ery. rhusiopathiae* was maintained for a sufficiently long period in 1 per cent. of peptone broth in Ringer's solution, and this was used as the diluting fluid in all subsequent experiments. The additional precaution was also taken of inoculating 50 per cent. of the control mice at the beginning of the series and the remainder at the end, so as to be able to observe any difference in virulence arising from the time factor.

The work on *Pasteurella aviseptica* was done in two stages, *viz.*, in Experiment 1 sulphathiazole, sulphamezathine and sulphanilamide were tested and in Experiment 2 sulphamezathine, sulphadiazine and sulphapyridine. With *P. boviseptica* (Experiment 3) sulphanilamide was omitted, the other four drugs being tested simultaneously. In all later experiments the five drugs were tested side by side.

Three levels of dosage were used, *viz.*, 18 mg. (1 mg. per g.), 4·5 mg. (0·25 mg. per g.), 1·1 mg. (0·0625 mg. per g.). In Experiments 1 and 2 the dosage was slightly higher, being 20 mg., 5 mg. and 1·25 mg. Treatment was carried out at 0 to 1 hour, and at 8, 24, 48, 72, 96 and 120 hours after inoculation, except where otherwise stated (Experiment 8). The drugs were made up in suspensions of gum tragacanth (50 per cent. of the B.P. strength) and the concentration of drug was so arranged that the dose in each case was contained in 0·4 c.c. of the suspension. Dosing was carried out by means of a 1 c.c. syringe attached to a blunt cannula which was passed down the oesophagus of the mouse. Dosing was carried out as expeditiously as possible so that not more than 30 minutes elapsed between the dosing of the first and the last mouse. The mice were kept under observation for a period of at least seven days after the last recorded death.

All mice which died were subjected to *post-mortem* examination and cultures were prepared from the organs and heart blood. Only when the inoculated organism was isolated by culture and identified was the death of the mouse in question attributed to the infection. Other deaths occasionally arose through faulty dosing or in some cases from poisoning by the drugs.

RESULTS

Experiment 1. *Pasteurella aviseptica* (Table I)

The strain used, P.5 (Kyaw), had been isolated from the heart blood of a case of fowl cholera in 1933 and kept in the dry state.

* This phenomenon was reported at a meeting of the Society for General Microbiology in July, 1945, and will be the subject of a separate publication.

The L.D.$_{50}$ for mice (saline suspension i.p.) was approximately 200 organisms, death taking place within 72 hours, and about ten times this dose was used (2,450 organisms). The growth of 18-hour cultures on blood agar slopes was washed off with saline, and the suspension standardised to Brown's tube 1, tenfold dilutions then being made in saline. Serum agar pour-plates were used for counting. It will be seen from Table I that in the group of mice which received the maximum dose of sulphanilamide three mice died after the second dose. They showed inco-ordination of the limbs, followed by paralysis and extremely slow respiration before death. There were no visible lesions at *post-mortem* examination, and no organism was isolated on culture. It was concluded that the mice were poisoned by the drug.

Only nine of the ten controls died in this experiment. Sulphamezathine proved completely effective in preventing deaths at all the levels of dosage used. Sulphathiazole was effective at only the two higher levels, while seven mice died in the lowest dose group. Sulphanilamide was not effective—one mouse died in the top dose group, six in the middle group, and ten in the lowest dose group.

TABLE I

EXPERIMENT 1. *Pasteurella aviseptica* (*P.5*)
Inoculum 2,450 organisms (approx. 10 × L.D.$_{50}$)

Drug	*Dose*	*Number of mice*	*Numbers dying on days*						*Total dead*
			1	2	3	4	5	6	
	Mg.								
Sulphanilamide	20	10	(3*)	—	—	—	—	1	1
	5	10	—	—	3	1	—	2	6
	1·25	10	1	2	2	4	—	1	10
Sulphamezathine ...	20	10	—	(1)	—	—	—	—	0
	5	10	(1)	—	—	—	—	—	0
	1·25	10	—	—	—	—	—	—	0
Sulphathiazole	20	10	—	—	—	—	—	—	0
	5	10	—	—	—	—	—	—	0
	1·25	10	—	2	3	2	—	—	7
None	None	10	3	3	3	—	—	—	9

* Mice died from toxic effects of drug.

Experiment 2. *Pasteurella aviseptica* (Table II)

The organism and procedure were as in Experiment 1. In this case the inoculum was 5,000 organisms which killed 100 per cent. of the control mice, as did also the dose of 500 organisms.

All the mice treated with the sulphadiazine 20 mg. dose died, nine of them in the first six days. No organism could be isolated from any of them at *post-mortem* examination, and it was concluded

that the dose of sulphadiazine had been toxic. No deaths occurred at either of the two lower dosage levels of sulphadiazine, indicating complete protection. One mouse died from infection in the sulphamezathine lowest dose group. Sulphapyridine protected the mice at the two higher levels of dosage, but four mice died at the lowest dosage level.

TABLE II

EXPERIMENT 2. *Pasteurella aviseptica* (*P.5*)
Inoculum 5,000 organisms (approx. 20 × $L.D._{50}$)

Drug	*Dose*	*Number of mice*	*Numbers dying on days after inoculation*										*Total dead*
			1	2	3	4	5	6	7	8	9	10	
	Mg.												
Sulphamezathine	20	10	—	—	—	—	—	—	—	—	—	—	0
	5	10	—	—	—	—	—	—	—	—	—	—	0
	1·25	10	—	—	—	1	—	—	—	—	—	—	1
Sulphadiazine	20	10	—	(2*)	(1*)	—	—	(6*)	—	—	—	(1*)	—
	5	10	—	—	—	—	—	—	(1)	—	—	—	0
	1·25	10	—	—	—	—	—	—	—	—	—	—	0
Sulphapyridine	20	10	—	—	—	—	—	—	—	—	—	—	0
	5	10	—	—	—	—	—	—	—	—	—	—	0
	1·25	10	—	—	1	1	—	1	—	1	—	—	4
None	None	10	9	1	—	—	—	—	—	—	—	—	10

* Mice died from toxic effects of drug.

Experiment 3. *Pasteurella boviseptica* (Table III)

The strain used, P.1 (Kyaw), had been isolated from a case of acute pneumonia and septicaemia in a calf and kept in the dried state.

It was found that this organism did not survive in saline, and dilutions of culture were made in Ringer's solution, after washing off serum agar slopes and standardising the opacity as in Experiment 1. It was also found necessary to use horse serum in making plates for counting as the organism failed to grow in bovine serum agar. This organism proved to be highly virulent. The $L.D._{50}$ was approximately eight organisms and death occurred within five days. Groups of 20 mice were inoculated with 16,320 organisms, *i.e.*, approximately 2,000 × $L.D._{50}$.

In the case of sulphadiazine the highest dose was reduced to 9 mg. (approximately 0·5 mg. per g.), because of previous toxicity, but some fatalities again occurred, apparently from this cause.

Sulphadiazine was the most effective drug as no deaths occurred at the lowest level of dosage (1·1 mg. per day). Sulphapyridine was effective at the two higher levels of dosage, but one death occurred in the lowest group. In the sulphamezathine groups no mice died at the highest level, one died in the middle group, and two in the

lowest group. In the case of the sulphathiazole treated mice, none died from infection at the top dose level, but one death occurred in the middle group and six on the lowest dose. There were 100 per cent. deaths in the controls.

TABLE III

EXPERIMENT 3. *Pasteurella boviseptica* (*P.1*)

Inoculum	*Drug*	*Dose*	*No. of mice*	*Numbers dying on days after inoculation*									*Total dead*
				1	2	3	4	5	6	7	8	9	
		Mg.											
16320	Sulphathiazole	18	20	—	—	(1)	(2)	—	(1)	—	—	—	—
		4·5	20	—	1	—	—	—	—	—	—	—	1
		1·1	20	—	2	—	1	—	2	1	—	—	6
16320	Sulphadiazine	9	20	—	—	—	(1*)	—	(2*)	(3*)	(1*)	(1*)	—
		4·5	20	—	—	—	—	—	—	—	—	—	0
		1·1	20	—	—	—	—	—	—	—	—	—	0
16320	Sulphamezathine	18	20	(1)	—	—	—	—	—	—	—	—	0
		4·5	20	—	1	—	—	—	—	—	—	—	1
		1·1	20	—	—	—	—	—	1	1	—	—	2
16320	Sulphapyridine	18	20	(1)	—	—	—	—	—	—	—	—	0
		4·5	20	—	—	—	—	—	—	—	—	—	0
		1·1	20	—	—	—	1	—	—	—	—	—	1
16320	None	None	20	—	16	—	4	—	—	—	—	—	20
816	None	None	20	—	8	6	2	1	—	—	—	—	17
81	None	None	20	1	8	3	1	—	—	—	—	—	13
8	None	None	20	—	6	1	2	—	—	—	—	—	9

* Mice died from toxic effects of drug.

Experiment 4. *Erysipelothrix rhusiopathiae*

The strain used (Elmslie) had been isolated from a case of swine erysipelas. A 24-hour broth culture was diluted in 1 per cent. peptone broth in Ringer's solution. The $L.D._{50}$ in mice consisted of a small number of organisms, possibly less than 20.* The majority of deaths occurred between the third and eighth days. The inoculum used in the experiments was 230,000 organisms (more than 1,000 $\times$ $L.D._{50}$.

The dosage of sulphadiazine in this experiment was reduced still further so that the 4·5 mg. dose was the highest, and a lowest dose of 0·28 mg. (0·0156 mg. per g.) was added for the third group. There was apparently no deaths from toxicity in this experiment, but none of the drugs had any effect on the course of infection, and all the mice (treated and controls) died within eight days. This con-

* In the experiment it was not accurately determined because the mice inoculated with the lowest dose (23 organisms) were males and fought with each other, possibly transmitting the infection and raising the number of deaths.

firms previous reports of the ineffectiveness of some of these drugs in *Ery. rhusiopathiae* infection in mice. (Klauder and Rule, 1944; Konst, 1945.)

Experiment 5. *Haemolytic Streptococcus Lancefield, Group C*

The strain used (S.34) had been isolated from a case of bovine mastitis some 15 years earlier and kept in meat broth. Eighteen-hour serum broth subcultures were used. The virulence of this strain which had been used in previous sulphanilamide tests by Stableforth *et al.* (1943) had to be raised. After eight mouse passages, it was found to be very high and had an $L.D._{50}$ of less than ten organisms, death occurring within 72 hours.

The drug treatment was quite unsuccessful in controlling this infection, presumably owing to its extreme virulence, and a table of results is not included.

Experiment 6. *Pasteurella aviseptica* (*P.5*)

The organisms was retested using 1 per cent. broth instead of saline as diluting fluid. The $L.D._{50}$ was between 116 and 1,160 organisms, the majority of deaths occurring within 48 hours. The inoculum used was 160,000 organisms (approximately 1,000 $\times$ $L.D._{50}$). Sulphapyridine treatment only was used. The results obtained confirmed those of Experiment 2, as the mice were protected from the infection by the two highest dose levels (18 mg. and 4·5 mg.), but not by the lowest dose (1·1 mg.).

Experiments 7 *and* 8. *Salmonella cholerae suis* (Table IV)

The strain (Lytle) had been isolated from a case of paratyphoid in a young pig and kept in the dried state. Eighteen-hour peptone broth cultures were made by subculture of a selected smooth colony of the organism from the surface of an agar plate. The cultures were diluted in 1 per cent. broth in Ringer's solution. The $L.D._{50}$ for mice proved to be between 40 and 400 organisms. Deaths did not occur before the fifth day after inoculation and continued for several days.

When drug treatment was started immediately after inoculation it was completed before deaths began to occur in the control group, and all the drugs entirely failed to arrest the infection. At the end of 14 days there was no difference between the control and treated groups as 80 to 100 per cent. of all mice had died.

When untreated mice were killed and examined during the five days immediately following inoculation, no organism could be isolated from the liver spleen or heart blood before the 29th hour, and large numbers were not found until the third day.

It was decided, therefore, to repeat the experiment commencing treatment on the third day after inoculation and continuing until the eighth day. The results of this experiment are shown in Table IV.

TABLE IV

EXPERIMENT 8. *Salmonella cholerae suis*

Inoculum	*Drug*	*Dose*	*No. of mice*	*Numbers dying on days after inoculation*										*Total 14th day*										*Total 24th day*	*Total 32nd day*
				5	6	7	8	9	10	11	12	13	14		15	16	17	18	19	20	21	22	23	24		
		Mg.																							
40,000	Sulphanilamide	18	10	—	—	—	1	—	1	1	1	—	1	5	2	—	1	—	—	—	—	—	1	—	9	9
		4·5	10	—	1	3	2	—	1	—	—	—	—	7	1	—	—	—	—	1	—	—	—	—	9	9
		1·1	10	—	—	1	—	4	—	2	—	—	—	7	—	1	2	—	—	—	—	—	—	—	10	10
40,000	Sulphathiazole	18	10	(1)	—	—	—	—	—	—	2	—	—	2	—	2	1	—	1	—	—	2	—	—	8	10
		4·5	10	—	—	2	1	1	1	—	—	3	—	8	—	—	1	—	—	—	—	—	—	—	9	9
		1·1	10	—	2	—	2	3	—	2	1	—	—	10	—	—	—	—	—	—	—	—	—	—	10	10
40,000	Sulphadiazine	4·5	10	—	—	1	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	2	3
		1·1	10	—	—	—	—	—	(1)	—	1	1	—	2	—	1	—	—	—	—	—	—	—	—	3	4
		0·3	10	—	—	—	—	—	1	1	1	—	2	5	—	—	1	—	—	1	—	—	—	—	7	7
40,000	Sulphapyridine	18	10	—	(1)	—	—	—	—	1	—	—	1	2	2	1	—	—	—	1	1	—	—	1	8	9
		4·5	10	—	—	—	—	—	1	—	1	2	2	6	—	1	1	—	1	—	—	—	1	—	10	10
		1·1	10	—	—	2	—	1	—	—	1	1	1	6	—	—	—	—	2	—	—	—	1	—	9	10
40,000	Sulphamezathine	18	10	—	—	1	—	—	—	—	—	—	—	1	1	2	1	1	1	—	1	—	—	—	8	8
		4·5	10	—	—	2	—	—	—	—	—	2	1	5	—	3	—	—	—	—	—	—	—	—	8	10
		1·1	10	—	3	1	1	2	—	2	—	—	—	9	—	—	—	—	—	—	—	—	—	—	9	10
40,000	None	None	10	—	2	2	2	—	3	1	—	—	—	10	—	—	—	—	—	—	—	—	—	—	10	10
4,000	None	None	10	—	3	1	2	—	1	—	2	—	—	9	—	—	1	—	—	—	—	—	—	—	10	10
400	None	None	10	—	—	—	2	1	4	—	1	—	—	8	—	—	—	—	—	—	—	—	—	—	8	8
40	None	None	10	—	—	—	—	—	—	—	—	—	—	0	1	—	—	—	—	—	—	—	—	—	1	3
4	None	None	10	—	—	—	—	—	—	—	—	—	—	0	—	—	—	—	—	—	1	—	—	—	1	1

Sulphadiazine was the most effective drug as the 4·5 mg. dose enabled seven of the ten mice to survive up to the 32nd day. There were four deaths on the 1·1 mg. dose, and seven deaths at the lowest dose level (0·28 mg.). Other drugs, in doses of 18 mg., had some delaying effect on death as at the 14th day, when all the controls had died, 80 to 90 per cent. of mice in the appropriate sulphapyridine, sulphamezathine and sulphathiazole groups had survived. Sulphanilamide was less effective. As the ten mice of each group were kept together in single cages the infection no doubt continued to spread amongst them, and at the end of a month 80 to 100 per cent. of all the mice had died, except in the sulphadiazine groups.

DISCUSSION

When comparing the efficiency of the different sulphonamide drugs in mice it must be remembered that the results are influenced by the metabolism of the drugs in the mouse. It is known, for example, that sulphadiazine produces blood concentrations in mice approximately four times those given by the same dose of sulphapyridine (Feinstone *et al.*, 1940), while sulphathiazole produces still lower levels which fall more rapidly between each dose. Factors of this kind would account for differences between *in vivo* and *in vitro* results. In addition, the metabolism of the drugs differs in different animal genera (Maclay and Slavin, 1947), and discretion must therefore be used in interpreting the results of *in vivo* experiments, especially with respect to comparisons between drugs.

SUMMARY

Mice in groups of 10 or 20 were experimentally infected by intraperitoneal inoculation with *P. aviseptica*, *P. boviseptica*, *Ery. rhusiopathiae*, a Group C Streptococcus, and *S. cholerae suis*. Treatment with sulphathiazole, sulphadiazine, sulphamezathine, sulphapyridine or sulphanilamide was given by intraoesophageal administration of suspensions for five days after inoculation. Three levels of dosage were used—approximately 1 mg. per g. per day, 0·25 mg. per g. and 0·0625 mg. per g.—except in the case of sulphadiazine, where doses were reduced in later experiments because of toxicity at the higher levels.

In the *P. aviseptica* infection sulphamezathine and sulphadiazine prevented deaths from infection at all three levels of dosage, but the latter was toxic to the mice at the highest dose of 1 mg. per g. Sulphapyridine and sulphathiazole were completely effective at the two higher dosage levels only, and sulphanilamide was relatively ineffective.

In the *P. boviseptica* infection only sulphadiazine prevented all deaths at the lowest dosage level (0·0625 mg. per g.). Sulphapyridine was completely effective at the two higher levels of dosage, but one death occurred at the lowest level. With sulphamezathine

one death occurred in the middle group, and two in the lowest dose group. Sulphanilamide was not tested.

Ery. rhusiopathiae infection was not controlled by any of the drugs in the doses used, nor was the infection by a strain of Group C streptococcus, the virulence of which had been raised by eight mouse passages.

In *S. cholerae suis* infection the highest dosage levels (1 mg. per g.) of sulphamezathine, sulphapyridine and sulphathiazole, delayed death of the mice but did not prevent it. Only sulphadiazine was effective in protecting mice throughout the period of observation (32 days), 70 and 60 per cent. surviving at dosage levels of 0·25 mg. per g. and 0·0625 mg. per g. respectively.

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We also wish to thank Dr. A. W. Stableforth for his help and encouragement while the work was being carried out.

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SULPHONAMIDE INVESTIGATIONS

III. The Activity *in vitro* of Sulphathiazole, Sulphadiazine, Sulphamerazine, Sulphapyridine, Sulphamezathine and Sulphanilamide on Some Common Animal Pathogens

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INTRODUCTION

The work reported here formed part of the series of investigations on the veterinary application of some of the newer sulphonamide drugs outlined in the first paper of this series (Maclay and Slavin, 1947), in which absorption, excretion and dosage were dealt with. The present observations concern the *in vitro* sensitivity of some common animal pathogens to sulphathiazole, sulphadiazine, sulphamerazine, sulphapyridine, sulphamezathine and sulphanilamide. The sensitivity of several of these organisms has also been tested in therapeutic experiments in mice (Slavin and Maclay, 1947).

In the earlier stages of these studies search was made for a simple laboratory method which would give consistent results with a wide range of bacteria. Most of the well-known *in vitro* methods were tried. Some were useful for studying selected organisms, but for a long time no common test was found suitable for general application. Ordinary culture media were ruled out by the presence of varying amounts of sulphonamide antagonising substances. Even if a satisfactory solid medium were found, pit and ditch methods were not regarded by us as satisfactory, owing to the difficulty of interpreting results due to the constantly changing concentration of drug in the medium. Fleming's slide-cell technique, using defibrinated blood as the culture medium, was restricted in range. A synthetic medium free from sulphonamide antagonists and of sufficiently simple nature to be of practical value, was available at the time only for the coli-typhoid group of organisms (Wood, 1942). A semi-synthetic medium containing hydrolysed casein and stated to be relatively free from antagonists (Lee, Epstein and Foley, 1943) appeared promising at first, as it supported the growth of a wide variety of bacteria, but experience revealed a lack of uniformity in different batches both in nutrient properties and antisulphonamide action. It was not until after the publication of a paper by Harper and Cawston (1945) that by adopting the method they have described we found *in vitro* testing satisfactory for our purpose.

We confirmed the claim of these authors that there is some property in lysed horse blood (treated as described in the original publication) which increases the activity of sulphonamide drugs in

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ordinary culture medium (nutrient agar) without being in any way detrimental to the growth of the organisms, and in fact supplying necessary growth factors for some of the more fastidious bacteria.

We found that cow blood treated in the same way did not possess this property. Harper and Cawston had already reported on sheep, rabbit and human bloods.

METHOD

The method of Harper and Cawston was only slightly modified. Twenty-five ml. quantities of agar were used for the plates. The drug solutions and lysed horse red cell solution prepared from oxalated blood were added to the molten agar at 50° C. before plates were poured. The final pH of the medium was approximately 7·5. In our experience 3 per cent. of the lysed red cell solution was sufficient to allow the maximum efficiency of the drugs, but as recommended 5 per cent. was employed in the tests to ensure good growth of fastidious organisms.

All six drugs were used at two concentrations, namely, 1 mg. per 100 ml. and 5 mg. per 100 ml. Up to 12 cultures were sown on a plate with a 2 mm. diameter loop. Approximately equal inocula were obtained by diluting 24-hour serum broth or peptone broth cultures either 100 times or 1,000 times in 10 per cent. broth-saline, according to a naked-eye estimate of their opacity.

TABLE I

GENERAL REVIEW OF ORGANISMS TESTED

Organism	*No of strains*	*Sulphathiazole*	*Sulphadiazine*	*Sulphamerazine*	*Sulphapyridine*	*Sulphamezathine*	*Sulphanilamide*
Str. agalactiae	14	S	S	S	S	S	S
„	1	R	R	R	R	R	R
Str. dysgalactiae	3	S	S	S	S	S	S
Str. uberis	3	R	R	R	R	R	R
„	1	S	S	S	S	S	S
„	1	I	I	I	I	I	I
Streptococcus Gp. C	4	S	S	S	S	S	S
„	1	S	I	I	I	I	I
Streptococcus Gp. G	4	S	S	S	S	S	S
C. pyogenes	11	S	S	S	S	S	S
„	1	R	R	R	R	R	R
S. aureus	5	S or I	R	R	R	R	R
Haemolytic coccobacillus	2	S	S	S	S	S	I
E. rhusiopathiae	1	R	R	R	R	R	R

S = Inhibited by 1 mg. and 5 mg. concentrations.
I = „ higher concentrations only.
R = Not inhibited by higher concentrations.

TABLE II

SUMMARY OF RESULTS OBTAINED IN THREE MOST RECENT TESTS

Organism	*Strain*	*Sulphathiazole*	*Sulphadiazine*	*Sulphamerazine*	*Sulphapyridine*	*Sulphamezathine*	*Sulphanilamide*	*Control*
Str. agalactiae	1768	—	—	—	—	—	—	+
„	3188	—	—	—	—	—	—	+
„	3187	—	—	—	—	—	—	+
C. pyogenes	B.11	—	—	—	—	—	—	+
„	C.1	—	—	—	—	—	—	+
Str. dysgalactiae	3174	—	—	—	—	—	—	+
P. septica	1877	—	—	—	—	—	—	+
„	Bythell	±	±	±	+	+	+	+
„	Bruford	—	±	±	±	±	+	+
„	P.22	—	—	—	±	±	+	+
„	P.5	—	±	±	±	±	+	+
S. aureus	P	±	+	+	+	+	+	+
„	FDA.209	±	+	+	+	+	+	+
S. cholerae suis	Lytle	—	—	—	—	±	+	+
„	Lewis	—	—	—	±	+	+	+
S. typhimurium	Doyle	±	±	±	±	+	+	+
S. pullorum	11	—	—	—	±	±	+	+
S. gallinarum	16	—	—	—	—	±	+	+
S. thompson	H.20	—	±	±	±	+	+	+
Bact. viscosum equi	3365	—	±	+	+	+	+	+
Bact. coli	231	—	—	—	±	±	+	+
Br. bronchiseptica	452	—	—	—	—	±	±	+
Haem. coccobac.	Barker	—	—	—	—	—	±	+
Actinobacillus lignieresi	4189	—	—	—	±	±	+	+
Listerella monocytogenes	2166	—	±	±	±	±	+	+
Str. equi	6179	—	±	±	±	±	+	+
C. ovis	3451	—	—	—	—	—	—	+

+ = Growth at both 1 mg. and 5 mg. per 100 ml. drug concentrations.
± = Growth at 1 mg. per 100 ml. only.
— = No growth at either concentration.

RESULTS

A general review of the results is shown in Table I. Other strains tested are shown in detail in Table II.

Frequent repetition of experiments with the same strains of bacteria yielded uniform results, but it was apparent that any slight variation in conditions, *e.g.*, in the pH of the medium, was likely to cause relatively marked differences in the results, particularly with

organisms of *intermediate* sensitivity, *e.g.*, the pasteurellae and *Bact. viscosum equi.*

DISCUSSION

The results of this test indicate that under the conditions of the experiments the growth of a number of animal pathogens is inhibited by many of the six sulphonamide drugs used. It should be noted that the test is one of bacteriostasis only, and that at the concentrations used the drugs have no bactericidal action.

By testing the six drugs simultaneously on each organism a clear picture is obtained of its degree of sensitivity, as the drugs range from the highly active sulphathiazole to the relatively slightly active sulphanilamide, and the position of an organism of intermediate sensitivity is at once apparent.

In vivo, the relative order of activity of the drugs may be altered by factors affecting their metabolism in the host. In treating pasteurella and *S. cholerae suis* infections in mice, for example, sulphadiazine has proved superior to sulphathiazole (Slavin and Maclay, 1947), although *in vitro* the latter is the more active drug.

CONCLUSIONS

The bacteriostatic activities of sulphathiazole, sulphadiazine, sulphamerazine, sulphapyridine, sulphamezathine and sulphanilamide have been tested on a number of common bacteria by the method of Harper and Cawston (1945). The results indicate that in this order the drugs show a decreasing activity. The most active drug had also the widest range of activity.

Of the organisms tested the following were found to be generally sensitive to all sulphonamides : *Str. agalactiae*, *C. pyogenes*, Streptococcus Gp. C, Streptococcus Gp. G, *Str. dysgalactiae.*

The following were usually completely resistant : *Ery. rhusiopathiae* and *Str. uberis* ; *Staph. aureus* was also resistant to all the drugs except sulphathiazole.

The majority of organisms fell into an intermediate category, but the number of strains tested in most cases was small. These organisms were : *Br. bronchiseptica*, *Bact. viscosum equi*, Haemolytic coccobacillus, *Pasteurella septica*, *Bact. coli*, *S. typhimurium*, *S. cholerae suis*, *S. pullorum*, *S. thompson*, *Actinobacillus lignieresi*, *Listerella monocytogenes*, *Str. equi.*

All these " intermediate " organisms were sensitive to sulphathiazole, resistant to sulphanilamide, and variously affected by the intervening drugs. *S. typhimurium* and one of the pasteurella strains

were at the more resistant end of the scale, while among the relatively sensitive were the haemolytic coccobacillus *Br. bronchiseptica*, *Bact. coli* and *S. cholerae suis*.

The value of the tests is discussed.

ACKNOWLEDGMENTS

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CONJUNCTIVITIS AND KERATITIS OF CATTLE AND SHEEP ASSOCIATED WITH THE PRESENCE OF "CELL-INCLUSION" BODIES

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INTRODUCTION

Conjunctivitis is not uncommon amongst cattle and sheep. The uncomplicated disease is not serious and rarely results in blindness, but it is of some economic importance because it tends to be complicated by keratitis or ulceration of the cornea and to become chronic. Little attention was paid to the aetiology of the condition until Coles (1931a) reported that the disease in sheep in South Africa, where in some years it affects up to 25 per cent. of the population, could be readily transmitted by the lachrymal discharge. He attributed infection to an organism which he identified in the epithelial cells and designated *Rickettsia conjunctivae*. His findings have been confirmed in different parts of the world. Thus Donatien and Lestoquard (1938) reported the disease in Morocco, Lafenêtre (1939) observed it in France, Johnson (1938) in America, and more recently Beveridge (1942) studied the infection in Australia where it is widespread. Subsequently Coles (1935) described similar organisms causing specific conjunctivitis in goats and cattle.

Consideration will be given to the disease as it affects sheep and cattle in this country.

I. The Disease in Sheep

Conjunctivitis is often observed in flocks in this country, and while it may arise from several causes the disease as described by Coles is frequently encountered, being particularly prevalent amongst lambs. Exposure during severe weather and concurrent diseases, such as joint-ill or parasitism, appear to be important predisposing causes.

The primary symptoms are a simple conjunctivitis with hyperaemia and profuse lachrymation. Recovery may occur without complications in a week, but in severe cases the course is prolonged and vascularisation of the cornea, followed by opacity and sometimes ulceration, develop. Pannus formation was also observed by Johnson (1938), and he commented upon the similarity of appearance between it and lesions in human trachoma. Hypertrophic granulations on the conjunctiva and cicatricial contraction of the eyelids are features of the chronic disease in sheep.

EXPERIMENTAL FINDINGS

Transmission experiments

The material used in this investigation was obtained from a typical outbreak of sheep conjunctivitis in East Anglia. The condition was readily set up by instilling a few drops of lachrymal discharge from affected sheep into the eyes of normal lambs, and the agent was maintained by serial passage through 12 sheep in this way. Similar attempts to infect cattle, rabbits and guinea-pigs were unsuccessful. The infective agent failed to induce a reaction when injected subcutaneously, intradermally or intravenously into sheep, and it was concluded that its tissue requirements were specific.

The period of incubation in the experimental disease was consistently 48 hours, and within a few days the condition became bilateral. The symptoms induced were usually of the uncomplicated type but some animals showed vascularisation of the cornea, sometimes associated with opacity and delayed recovery. Under experimental conditions the disease spread rapidly to normal sheep placed in contact with affected animals. In one experiment, however, sheep were penned in groups with solid divisions 4 ft. high between them. When infection was introduced into one unit, it remained confined to the sheep in immediate contact with the infection. This would seem to indicate that transmission is direct and probably by droplet infection, the infective agent passing down the lachrymal ducts. Beveridge (1942) pointed out that in Australia the disease is much less common in winter than at other seasons and he suggested that flies might be responsible for its transmission. In this country it is more frequently observed during early spring when flies are not active, and crowding together of sheep would seem to be a more important factor in its spread.

It was found that sheep were infective not only at any stage of the disease but even after recovery, for transmission was successful using the conjunctival scrapings from two sheep 40 and 120 days respectively after the symptoms had disappeared. Further tests revealed, however, that it was not possible to set up the disease with pooled material taken from four sheep approximately 270 days after recovery. These results, which are in accordance with those of Beveridge (1942), imply that " carriers " are present in infected flocks and must obviously play an important role in the spread of the disease.

Immunity to Infection

Beveridge carried out immunity tests on 19 recovered sheep and found that they were resistant to experimental infection 100 days after infection. In a few cases immunity persisted for 406 days. In the present investigation it was not possible to undertake extensive experiments on this aspect of the problem, but one recovered sheep was immune to a subsequent dose of infective lachrymal

secretion 24 days, and another 48 days after apparent recovery. While it is evident that a considerable degree of immunity may follow a clinical attack of the disease, this does not always happen since relapses are common, especially if sheep are subjected to adverse conditions. It is also probable that the organism persists in the eye of the immune animal and thus may be transmitted to in-contact healthy stock ; this would explain why lambs not infrequently show symptoms although the ewes are not affected.

The Infective Agent

The agent would not pass through Berkefeld V filters. Lachrymal secretion was infective after standing in the ice-chest for two days, but was inactivated in the same period by 50 per cent. glycerine and when dried over P_2O_5. The resistance of the sheep used for these experiments was subsequently tested by the use of known virulent material.

The inclusion bodies described by Coles (1931a) were demonstrated without difficulty. The most suitable method was to lightly curette the conjunctiva, care being taken to avoid haemorrhage. Smears prepared from the scrapings were fixed with methyl alcohol and then stained with Giemsa diluted in buffer solution so that each c.c. contained two drops of stain. The stain was allowed to act for two hours and the preparation was then differentiated with ethyl alcohol. By this method the inclusion bodies stained a pale bluish purple. Several other stains were used, but did not serve to differentiate the bodies from the cell cytoplasm more distinctly. With Castaneda's method (1930) they stained blue, and by the modification of Macchiavello's method recommended by Begg *et al.* (1944) they were red, so that they did not react like Rickettsiae to either of these stains. They were Gram negative.

In clinical cases the superficial cells of the conjunctiva contained large numbers of ring-like round or oval bodies 0·5 to 1μ in diameter (Fig. 1). Similar structures were found lying between the cells and they were readily distinguished from bacteria by the paleness of their staining. They were often found in such large numbers as to cause bursting of the cell membrane, but usually the structure of the cells was not altered as a result of their presence. The organisms appeared to be limited by a well-marked chromatin ring which in some was thickened at the poles, giving a bipolar appearance. The extracellular ring forms often attained a size of 2μ in diameter and in some cases larger bodies 3 to 8μ in diameter (Fig. 2) with a granular centre, were also present. These granular bodies appear to be " initial bodies," since minute coccal forms undergoing transformation into bipolar bodies were often seen emerging from them. The blue ring structures which were mainly intracellular and present in large numbers in acute infection, are comparable to elementary bodies of virus diseases. Repeated examinations have revealed that these

ring-like bodies decrease in number but do not altogether disappear with clinical recovery, although the initial bodies persist.

It seems obvious that the organism has a developmental cycle but it is difficult to establish the sequence of events from the examination of a surface so contaminated as the conjunctiva. Moreover, the presence in the normal eye of the intracellular bodies, described by Coles (1931b) as a harmless parasite of sheep, complicates the position still further, although these structures are much larger and stain deeper than those which characterise conjunctivitis and in their typical form they can be readily distinguished. Beveridge (1942) failed to cultivate rickettsiae from infected eyes on the chorio-allantoic membrane, yolk sac and amniotic fluid of developing chick embryos. Negative results were also obtained using Zinsser's (1939) tissue culture-agar containing embryonic conjunctival tissue. Such endeavours are of course rendered difficult by the contaminated state of the infective material available. In this investigation an unsuccessful attempt was made to infect mice by intraperitoneal injection and cultivation in tissue culture media also failed.

DISCUSSION

Conjunctivitis associated with the presence of inclusion bodies in the conjunctival epithelium is not uncommon amongst sheep in this country. It usually manifests itself as a mild condition, but occasionally severe outbreaks occur. Recovered animals are carriers of infection and may be immune, but they are responsible for the persistence of the disease in flocks. Natural transmission probably arises through droplet infection, diseased sheep excreting the infection *via* the nasal cavity. Insects do not seem to play any part in its epidemiology.

The bodies found in the conjunctival cells are so intimately associated with the disease that they may be regarded as aetiologically significant. Coles identified them as rickettsia and proposed the name *R. conjunctivae*. They do not, however, conform altogether with the definition of the group, since such organisms are usually referred to as " small, often pleomorphic, Gram-negative, bacterium-like organisms, living and multiplying in arthropod tissues and behaving as obligate intracellular parasites and staining lightly with aniline dyes." Their morphology is not unlike *R. ruminatum*, which causes heartwater, and the larger number of organisms present in the cytoplasm is also rather characteristic of a rickettsial infection. In fact, only the absence of a known arthropod vector would exclude the organism being grouped with the Rickettsiae. Certain of its characters suggest that the infecting agent is of the nature of a virus. The inclusion bodies are not unlike those of psittacosis. The infective agent, like psittacosis virus, did not survive in 50 per cent. glycerine and both agents differ in this respect from many viruses. It seems probable that the inclusion bodies associated with con-

junctivitis represent an infective agent which cannot be classified with the viruses or rickettsiae, resembling both in some respects and probably occupying an intermediate position.

II. The Disease in Cattle

Coles (1933, 1936) demonstrated that a Rickettsia-like organism was associated with conjunctivitis in South African cattle, but although he was able to transmit the organism to normal bovines the typical symptoms of the disease were not reproduced. There are no references to a similar disease in this country although cases of keratitis do occur. Craig and Ratter (1940) investigated an outbreak which was attributed to a Friesian cow imported from Canada. They were unable to transmit the disease with ocular discharge obtained from an early case or mixed cultures from infected eyes and in this respect the disease resembled the South African condition. More recently, Dalling (1944) described the development of keratitis in consignments of young cattle on their arrival in this country from Northern Ireland. He found that the affected eyes showed different degrees of involvement, varying from a simple conjunctivitis to a condition in which the anterior chamber of the eye was filled with blood. He was much impressed by the rapidity with which even the most severely affected eyes become normal without any special treatment. By instilling the conjunctival secretion from affected animals into the eye of a normal bullock, Dalling was able to set up a mild form of the natural disease.

In this investigation material from two outbreaks of kerato-conjunctivitis was examined with the object of determining whether inclusion bodies described by Coles were a feature of the disease.

Outbreak 1.—The disease was noticed in October amongst a group of 20 heifers at pasture. They were then brought into a yard and the condition spread, soon affecting them all. The symptoms were those of a bilateral conjunctivitis with profuse lachrymation and intense irritation. There was little corneal involvement and complete recovery took place within 16 days. It was suggested that fly transmission was responsible for its dissemination at pasture, but the time of the year and its further spread when the cattle were housed was against this view. There was no previous history of the disease on the farm.

Smears from affected heifers revealed inclusion bodies in the conjunctival-epithelial cells.

Outbreak 2.—The death of calves on a dairy farm was reported, with the history that the animals became unthrifty and blind when about six weeks old. The total stock of eight was affected and all died before they were three months old. The ocular condition to which the deaths were attributed was severe, there was marked keratitis, corneal ulceration and impaired vision. The clinical appearance was suggestive of xerophthalmia, and some confirmation of this was obtained from an examination of the blood of one specimen, the serum vitamin A level being 20 I.U. per 100 ml. and carotene value 17 I.U. per 100 ml. On autopsy the liver of this animal was found to contain only 0·6 I.U. per g. of vitamin A, and there were

hyperplastic changes in the epithelial surfaces particularly of the rumen and bladder, with no other significant lesions. Reliable information regarding the diet fed to these calves was not available but it was obviously poor and milk had been allowed only for the first few days of life.

Smears of the conjunctival epithelium revealed that a small number of cells contained inclusion bodies.

EXPERIMENTAL FINDINGS

Transmission Experiments

Lachrymal discharge was collected from one affected animal (outbreak 1) and instilled within six hours into the eye of a normal three-month-old calf. Four days afterwards a mild conjunctivitis developed, evidenced by considerable irritation and profuse lachrymation. Inclusion bodies similar to those found in the natural disease were demonstrated, but they disappeared two days after the onset of symptoms. The condition became bilateral but rapidly improved and the animal was normal within seven days. An attempt to transmit the condition to a second calf with material obtained on the fifth day of the disease was unsuccessful.

Following the demonstration of inclusion bodies in outbreak 2, an attempt was made to transmit the disease to a four-month-old calf by the instillation of 1 c.c. of a mixture of fresh lachrymal discharge and conjunctival scrapings into the left eye. Four days later there were signs of irritation and on the fifth day a profuse lachrymation and conjunctivitis developed. The disease became bilateral four days after the left eye was first affected, but improvement quickly followed and recovery took place in ten days. Inclusion bodies were demonstrated in large numbers during the early stages of the disease, but were more difficult to detect as the ocular condition improved and disappeared altogether with recovery. Lachrymal discharge from this animal was taken during the early stages of the disease and instilled into the eye of a three-day-old calf, and again conjunctivitis, associated with the presence of similar inclusion bodies, resulted four days afterwards. The symptoms, however, in this animal were very mild and transitory, complete recovery taking place in three days. Calves were not available for further transmission experiments.

An attempt was made to precipitate a relapse in the first calf infected with material from outbreak 2, by introducing one drop of a 20 per cent. solution of ammonium hydroxide into the eye one month after recovery. This treatment was followed immediately by a simple conjunctivitis which persisted for three days, and smears of the conjunctival epithelium on the second day revealed the inclusions which characterised the first attack.

The "Cell-inclusion" Bodies

The methods used for demonstrating the inclusion bodies in sheep were employed, but the stain was not allowed to act for so

long as it was found that there is a tendency for the cell cytoplasm in bovines to stain deeply and obscure the inclusions. Coles (1936) has reported a similar finding.

The appearance and arrangement of the bodies found was essentially the same as described by Coles. They were mainly intracellular and not present in such large numbers as in sheep (Fig. 3), whilst affected cells remained unaltered. Most of the bodies were round, 0·2 to 0·6μ in diameter, whereas others were ovoid and bipolar. Most of the round forms stained evenly, but some had a ring-like appearance. Staining was deeper than in the case of the bodies found in sheep conjunctivitis.

The arrangement of the inclusion bodies within the cells was characteristic (Fig. 4), for they were aggregated together in groups of various sizes. Granular bodies 5 to 6μ in diameter and corresponding to the initial bodies seen in the sheep, were also present and there was evidence that the rounded forms developed from them. These initial bodies sometimes occurred singly and were always in the cytoplasm, differing in this respect from those observed in sheep which were mostly extracellular.

DISCUSSION

It seems probable that a number of specific infective conditions affecting the superficial structure of the eye may occur in bovines but, as indicated by the work of Coles, one form of the disease is characterised by the presence of intracellular inclusion bodies in the conjunctiva. The same condition has now been recognised in this country. Whether or not it is identical with contagious ophthalmia referred to in American literature, and attributed to *Haemophilus bovis* infection (Baldwin, 1945) must await further investigation but the severe cases observed in outbreak 2 would appear to be very similar clinically.

Unfortunately, the evidence obtained does not prove that the disease investigated was due to a specific infection, for in the transmission experiments carried out only a mild reaction was induced, and on the second passage it showed signs of decreased intensity. Such a result might be attributed to secondary bacterial infection, and it could even be argued that the severe reaction in outbreak 2 was due to bacterial infection following xerophthalmia. The appearance of inclusion bodies in the eyes of the experimental animals is, however, not without interest, since they characterised the disease in the two outbreaks and were a feature of the experimental condition. These inclusion bodies were numerous only during the early stages and disappeared before recovery was complete. They must, however, be capable of survival in small numbers or in some unrecognisable form because they reappeared in the eye of an animal which was subjected to irritation one month after clinical recovery. As in the case of sheep conjunctivitis, their presence may be aetiologically significant.

Coles (1933) also found that it was impossible to reproduce the severe ophthalmia, which was a feature of his outbreaks, although he too demonstrated that the inclusion bodies could be transmitted. The possibility that predisposing causes are concerned in the natural disease cannot be disregarded. It has been suggested by Bermasconi (1941, 1943) that nutritional deficiencies, and particularly vitamins A and D, are important aetiological factors in human trachoma, and this was the indication in outbreak 2 of this investigation.

The occurrence of the disease during or immediately following transit is a matter of some practical importance, and in this case it would seem that dietetic factors can be excluded. The conditions during shipping, however, favour a rapid spread of infection, especially as animals from a number of sources are collected together. The possibility that the irritant nature of the atmosphere of the holds, resulting from the manure and deficient ventilation, may act as a stimulus and precipitate a latent infection, must also be considered especially in view of the evidence that somewhat similar treatment caused the reappearance of inclusion bodies.

The comparative aspect of the conjunctivitis in sheep and cattle is interesting, since inclusion bodies believed to be aetiologically significant have been demonstrated in humans suffering from trachoma, ophthalmia neonatorum and so-called swimming bath conjunctivitis. There seems no reason to doubt that these bodies, which show species specificity, are characteristic of conjunctival infections in a number of animals. In particular, the disease in cattle shows certain points of resemblance to trachoma, for the early disappearance of inclusion bodies observed in the bovine disease is a feature of trachoma (Bland, 1944), and this condition shows the same low infectivity (Julianelle, 1938).

SUMMARY

A specific conjunctivitis of sheep characterised by the presence of inclusion bodies in the superficial cells of the conjunctiva is not uncommon in this country. It is usually a mild and transitory condition, but may be complicated by keratitis and become chronic. Some immunity follows infection, as shown by the fact that two sheep were found to be resistant 24 and 48 days after recovery. Carriers were demonstrated by showing that the lachrymal secretion of a sheep was infective 120 days after recovery. The causal agent was infective in lachrymal discharge stored for two days in the ice-chest, but was inactivated in the same period by 50 per cent. glycerine and when dried over P_2O_5. The nature of the inclusion bodies which characterise the disease is discussed.

Two outbreaks of bovine kerato-conjunctivitis were studied. Conjunctivitis was reproduced with material from both cases, but the induced condition was relatively mild and may have been due to transferred secondary bacterial infection. Conjunctival inclusion

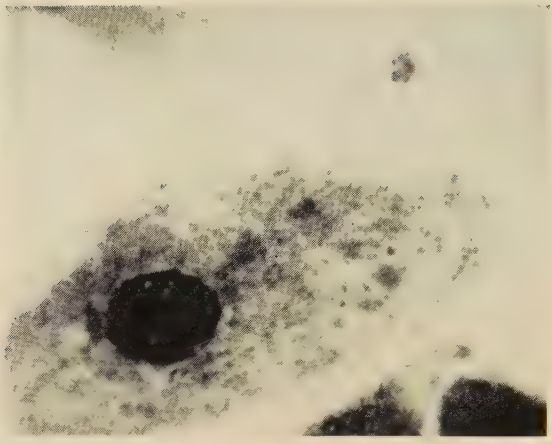

FIG. 1.—Sheep. Conjunctival cell, showing a large number of inclusion bodies. x 1200.

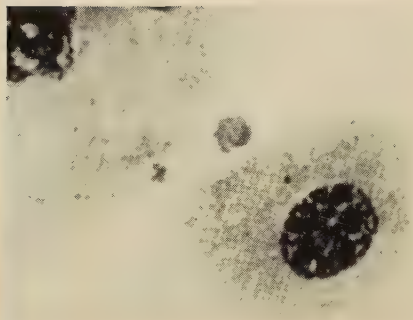

FIG. 2.—Sheep. Extracellular initial body situated between two heavily infected epithelial cells. x 950.

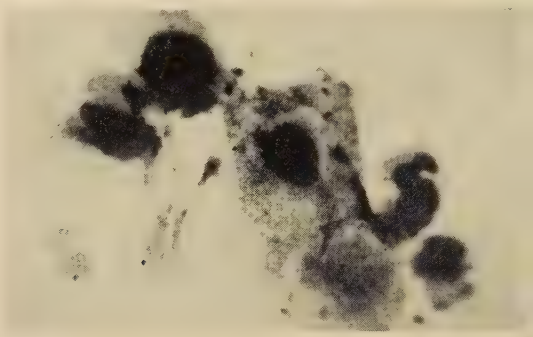

FIG. 3.—Bovine. Group of conjunctival cells showing inclusion bodies. x 650.

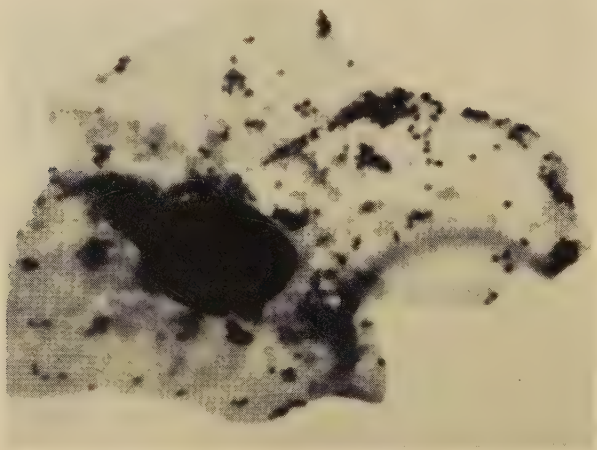

FIG. 4.—Bovine. Conjunctival cell showing the characteristic arrangement of inclusion bodies. x 1570.

bodies were found in large numbers but only during the early stages of the natural disease and they were also present in the experimental subjects. Consideration is given to the factors which may predispose to the disease.

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SOME OBSERVATIONS ON PEROSIS IN CHICKS

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INTRODUCTION

In previous publications (Bruce *et al.*, 1946 a and b) we recorded the occurrence of perosis among chicks used for the assay of vitamin D_3, and also described certain radiographic features which make it possible to recognise the disease at an early stage of development. While these papers were in press, experiments were in progress designed to show whether the addition of manganese to the basal diet of the chicks prevented the disease. These experiments, to which a brief reference was made in a footnote to the previous publication (Bruce *et al.*, 1946b), are described more fully here.

The aetiology of perosis is complex and typical symptoms and lesions of the disease can be evoked by deficiencies of different factors in the diet; manganese is only one of them.

EXPERIMENTAL

The effect of added manganese on the incidence of perosis among birds receiving the Olsson diet

The perosis observed by Bruce *et al.* (1946b) occurred with two different diets, but the observations related mainly to the behaviour of birds which received the diet of Olsson (1941).

Though choline was not determined, this diet must have contained appreciable quantities of it supplied by the soya meal and brewers' yeast. The latter would also contain the various other factors of the vitamin B_2 complex. On the other hand, the manganese content of the diet, determined by the method of the Association of Official Agricultural Chemists (1940), was only 20 p.p.m. A deficiency of manganese therefore seemed the most probable cause of the occurrence of perosis among our chicks. Additions were made to the diet of 0·02 per cent. manganese sulphate, with and without 0·2 per cent. of choline chloride, and the effect was noted on the incidence of perosis. For assessment of perosis in these experiments, a standard set of radiographs was compiled showing varying degrees of severity of the disease (Fig. 1).

While it seems reasonably clear that stages (*c*) to (*f*) in fact represent different degrees of the slipped tendon disease (cf. Bruce *et al.*, 1946b), the inclusion of the first stage (*b*) is more speculative

(cf. p. 235) and therefore no bird was considered to have perosis unless the changes were at least as severe as those shown in radiograph (*c*) of the series.

To avoid the complication of rickets, without departing from the conditions in the earlier study, all the chicks were given the highest dose of cod liver oil used in routine assays. This amounted to about ¼ per cent. and supplied from 20 to 25 British Standards Institution (1940) units per 100 g. food.

On three occasions groups of approximately 24 newly-hatched chicks received for four weeks either the unsupplemented diet of Olsson (1941) or the diet with additions of (1) 0·02 per cent. $MnSO_4 \cdot 4H_2O$, (2) 0·2 per cent. choline chloride or (3) 0·02 per cent. $MnSO_4 \cdot 4H_2O$ and 0·2 per cent. choline chloride. At the end of that time the birds were examined clinically and radiographically for perosis.

Table I shows the results of this experiment. "Student's" (1908) *t*-test shows that the incidence of perosis fell significantly in the two groups which received manganese ($P << 0{\cdot}001$). The

TABLE I

NUMBER OF CHICKS SHOWING PEROSIS AT THE FOURTH WEEK OR EARLIER. (FIGURES IN PARENTHESIS DENOTE NUMBER OF CHICKS IN EACH GROUP)

Hatch number	*Olsson diet*	*Olsson diet + Mn*	*Olsson diet + choline*	*Olsson diet + Mn + choline*
68	5 (24)	0 (24)	9 (24)	1 (24)
70	7 (21)	0 (23)	1 (23)	0 (22)
71	7 (24)	0 (23)	5 (24)	0 (23)
Total	19 (69)	0 (70)	15 (71)	1 (69)
Percentage	27·5	0	21·1	1·5

difference between the group supplemented with choline and the unsupplemented group was not significant ($P = 0{\cdot}4$). There was no difference in the growth of the birds in the different groups.

The tarso-metatarsal distances of the birds which received manganese were significantly greater than those of the birds free from perosis on the unsupplemented diet or in the group which received choline alone. It is possible (cf. p. 235) that in the no-manganese group some of the chicks "free" from perosis as judged by the arbitrary standard were in fact suffering from a very mild form of the disease, and that slight swelling of the metatarsals may have occurred resulting in a smaller T.M.T. measurement.

As a result of these findings, manganese was incorporated into the basal diet as a routine measure, and 2,000 lb. of diet containing 0·02 per cent. of added manganese sulphate, sufficient to last for four hatches of about 250 chicks each, were prepared.

Assays of various oils and vitamin D_3 concentrates were carried out in the usual way (Olsson, 1936). All birds were examined for perosis at the fourth week as in the previous experiment. Results were compared with those obtained from four hatches of chicks which, at a corresponding season of the previous year, received a similar batch of diet without added manganese. Only three out of 961 birds (0·31 per cent.) which received the supplemented diet developed perosis, as against 27 out of 1,045 (2·57 per cent.) which had no added manganese. The difference between these two figures is highly significant ($P << 0{\cdot}001$). Although the two groups were not strictly comparable because they were not observed simultaneously, the results are supported by the fact that a return to the unsupplemented diet was followed by a marked increase in the incidence of perosis (p. 235).

The addition of 0·02 per cent. of $MnSO_4 \cdot 4H_2O$ to our diet increased its manganese content from about 20 to about 70 p.p.m.

We are informed by Professor Nils Olsson that he has not observed perosis in his strain of White Leghorn chicks either with the original Olsson diet or with modifications of it, although it should be noted that these modifications were only used for chicks up to three weeks of age. The original diet contained about 60, but the modifications only 25 to 35 p.p.m. of manganese.

Identification of early symptoms of perosis

Although it is almost impossible to recognise perosis by external appearance alone unless the tendon has slipped, radiographic studies of the hock joint allow of its diagnosis at an earlier stage. The radiographic appearance of the hock joint of birds suffering from perosis has already been described (Bruce *et al*, 1946b).

In the course of routine examination of radiographs of the hock of four-week-old birds, bones were seen which did not appear quite normal. In these bones the ends of the metatarsals were very slightly swollen and, occasionally, the shaft of the metatarsal was somewhat bent. Fig. 1 (*b*) is a typical instance. It was thought that this might be an early stage or a mild form of perosis, and an attempt was made to follow the development of hock joints showing these changes.

A group of 36 chicks was given the Olsson diet, including $\frac{1}{4}$ per cent. cod liver oil, and a similar group received the same diet supplemented with 0·02 per cent. manganese sulphate. Both hock joints of each bird were radiographed weekly beginning at the fourth week, and examined for perosis, the degree of perosis being recorded as follows: 0. No perosis. 1. Suspected perosis (radiograph (*b*) in Fig. 1). 2. Perosis without slipped tendon (radiographs (*c*) and (*d*) in Fig. 1). 3. Perosis with slipped tendon (radiographs (*e*) and (*f*) in Fig. 1).

No perosis appeared in any of the birds which received supplements of manganese except for a score of 1 in four of the birds,

and this was observed on not more than one occasion in any one bird. As the chicks grew, it became necessary, owing to space limitation, to discard from both groups birds which had shown no signs at all of perosis ; ten birds in each group were kept until the 20th week of age.

Table II shows the records up to the eleventh week of the birds receiving the Olsson diet not supplemented with manganese. Twenty of them showed some degree of perosis at some stage of the experiment. No fresh cases of perosis appeared after the seventh week. One each of the first and second degrees of perosis developed into the third, but in other cases the abnormality disappeared again with-

TABLE II

DEGREE OF PEROSIS SHOWN IN RADIOGRAPHS OF THE HOCK JOINT OF BIRDS RECEIVING THE OLSSON DIET NOT SUPPLEMENTED WITH MN.

(The method of scoring is described on page 234)

	Age in weeks							
Bird No.	4	5	6	7	8	9	10	11
635	0	0	0	1	0	0	0	0
901	0	1	1	0	0	0	0	0
903	0	0	0	0	0	0	0	0
904	2	1	0	1	0	0	0	0
905	0	0	0	0	0	0	0	0
906	1	2	0	0	0	0	0	0
908	0	0	0	0				
909	3	3	3	3	3	3	3	3
913	1	1	0	1	0	0	0	0
916	0	0	0	0				
917	0	0	0	0				
919	0	2	2	1	0	1	2	0
921	0	0	0	0				
923	2	0	0	0	0	0	0	0
924	0	0	1	2	0	0	1	0
925	0	0	0	0				
927	0	0	0	0				
930	0	0	0	0				
932	0	1	0	0	0	0	0	0
622	2	1	0	0	0	0	0	0
935	2	3	2	1	2	2	1	1
779	0	0	1	0	0	0	0	1
939	0	0	0	0				
942	3	3	3	3	3	3	3	3
943	0	0	0	1	2	2	2	1
944	0	2	2	3	3	3	3	3
946	0	0	0	0				
947	0	0	0	0				
948	0	0	0	0				
949	0	0	0	0				
651	0	0	0	0				
954	0	0	0	1	3	3	3	3
656	2	0	0	0				
956	0	0	0	0	0	0	0	0
957	1	2	1	3	2	2	2	1
699	1	0	2	0	2	1	2	1

out slipping of the tendon occurring. Five of the first degree cases developed into the second. After about the eleventh week the hock joint was so far developed that it became impossible to recognise the less severe stages of perosis, and only birds with slipped tendon showed abnormal radiographs.

The number of cases is not sufficient for very definite conclusions but these results suggest that the changes shown in Fig. 1 (*b*) are those of mild or early perosis.

The effect of perosis on the time of fusion of the elements of the hock joint

Nielsen (1942) has suggested that in perotic chicks the lines of ossification are open later than usual. Previous work reported from this laboratory (Bruce *et al.*, 1946b) gave no indication that the fusion of the hock joint was delayed in perotic birds, but this could not be finally verified until observations could be included with a diet on which chicks failed to develop the disease. From the birds used in the foregoing experiment, ten were chosen from the non-supplemented group in which perosis was definitely present (severity at least as in Fig. 1 (*c*)), and ten from the manganese supplemented group in which perosis was completely absent. Weekly radiographs of both legs were made until the 20th week, and the times of fusion of the elements of the hock joint were recorded in four stages : (1) fusion of the upper tarsals with each other ; (2) fusion of the upper tarsals with the tibia ; (3) attainment of full width by the lower tarsal ; (4) fusion of the lower tarsal with the metatarsals.

The results are recorded in Table III. The last stage had not been reached by any of the birds when the experiment ended at the 20th week, although one of the manganese supplemented birds showed almost complete fusion. There was no difference in time of fusion between the two groups at any stage, except for some delay in grossly deformed legs where it was mechanically impossible for fusion to occur.

DISCUSSION

The results of these experiments show that addition of Mn to the Olsson diet was almost entirely successful in preventing perosis in the chicks used for the assay of vitamin D_3. That choline was ineffective in preventing perosis under these conditions suggests that the choline content of the diet was already adequate and that Mn was the limiting factor. Recent work suggests that a deficiency of factors other than Mn and choline, *viz.*, biotin (Jukes and Bird, 1942, and Patrick *et al.*, 1942), nicotinic acid (Briggs, 1946, and Jukes *et al.*, 1947), riboflavin (Patrick *et al.*, 1944, and Bird *et al.*, 1946), and folic acid (Daniel *et al.*, 1946), may give rise to perosis. There is thus an indication that the aetiology of the disease is complex and that a variety of dietary factors is necessary in the enzyme

systems concerned in normal bone formation. With the exception of Mn and possibly choline, diets of natural foodstuffs are less likely to be lacking in these factors than purified diets with which the possible role of the other factors in the production of perosis has been demonstrated. It is as yet too early to form a complete picture of the chemical changes occurring in the growing bones of the hock joint of the young chick at the time when the rudimentary tarsals are fusing with the tibia and metatarsus to form the simple hinge

TABLE III

TIME, IN WEEKS, OF FUSION OF THE ELEMENTS OF THE HOCK JOINT

Olsson diet

Bird number	*Upper tarsals*		*Lower tarsals*	
	Fusion with each other	*Fusion with tibia*	*Attainment of full width*	*Fusion with metatarsals*
909	10	18	12	Not complete at 20 weeks
923	9	16	11	
932	9	15	11	
935	10	18	13	
942	11	Dead before sealing	14	
943	10	17	13	
944	10	Dead before sealing	13	
954	11	19	13	
957	9	17	12	
699	11	19	14	
Mean time of fusion	10	17	13	

Olsson diet + 0·02 per cent. manganese sulphate

Bird number	*Upper tarsals*		*Lower tarsals*	
	Fusion with each other	*Fusion with tibia*	*Attainment of full width*	*Fusion with metatarsals*
841	10	18	12	Not complete at 20 weeks
842	9	16	13	Almost sealed at 20 weeks
668	10	19	14	Not complete at 20 weeks
844	11	17	12	
848	10	17	12	
853	12	18	13	
854	12	Dead before sealing		
856	10	16	12	
858	10	16	12	
859	11	18	12	
Mean time of of fusion	11	17	12	

joint of the adult bird. The application of biochemical studies to fresh bone sections is likely to be of value in further elucidating the problem.

Beyond the fact that disordered bone formation in the hock seems to be present in perosis the exact changes which cause the enlargement and deformity of the hock have not been determined. Radiographic studies make it possible to detect the changes in incipient perosis at a time when the recognition of the disease by mere inspection and palpation is not possible. The observations of Schaible *et al.* (1944) that confinement in small cages with wire floors can cause a perosis-like condition, while birds on the same diet but not so confined remain normal, suggests that mechanical factors are of importance.

The localisation of the characteristic lesions to the hock joint and their occurrence at a particular period of the development of the bird—usually between three and six weeks of age—suggests that the structural changes which occur at this site, aggravated by the mechanical stresses imposed by rapidly increasing body weight and wire mesh floors, render the hock particularly liable to deformities when even slight disturbances of the bone-forming mechanism occur.

SUMMARY

The addition to the Olsson rachitogenic diet of 0·02 per cent. $MnSO_4 \cdot 4H_2O$ with or without 0·2 per cent. choline chloride caused a significant reduction in the incidence of perosis, whereas addition of 0·2 per cent. choline chloride alone had no effect.

In the course of routine assays of vitamin D_3 in various oils and concentrates, the incorporation of 0·02 per cent. $MnSO_4 4H_2O$ into the Olsson diet reduced the incidence of perosis from 2·57 to 0·31 per cent.

Weekly radiographic examinations over a period of 20 weeks of chicks with slightly abnormal hock joints, showed that in some birds these abnormalities became more severe and finally developed into typical perosis. In other birds the changes slowly disappeared and the hock became normal.

The time of fusion of the bones of the hock was the same in perotic birds as in those not suffering from the disease.

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FIG. 1.—Antero-posterior radiographs of the tarsus of four-week chicks, showing the stages in the development of perosis.

(*a*) Normal tarsus.
(*b*) - (*f*) Perosis, from the earliest stage with merely some blurring and swelling of the head of the metatarsus (*b*) to perosis with "slipped tendon" and bent metatarsus (*e* and *f*).
(*g*) Perosis and rickets.
(*h*) Rickets.

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A SEDIMENTATION METHOD FOR THE DETECTION AND COUNTING OF FASCIOLA EGGS IN FAECES

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INTRODUCTION

The methods normally used for the recovery and concentration of nematode eggs from faeces are unsatisfactory for those of trematodes, as the latter will not float in concentrated solutions of salt or sugar. *Fasciola* and paramphistome eggs are particularly difficult to deal with, because they burst so readily when placed in concentrated solutions. Although they will float in waterglass solutions, they are almost invariably split and so distorted as to be unrecognisable. On the other hand, fluke eggs sink more quickly in water than the majority of the finer faecal particles or any nematode eggs with which they are mixed. Advantage has been taken of this fact in designing the following simple apparatus for examining sedimented faeces layer by layer, both for diagnostic purposes and for making exact counts of fluke eggs in weighed amounts of faeces.

APPARATUS

The apparatus, which is drawn to scale in Fig. 1, is essentially a tap of special design made of brass, although glass or some plastic material such as Perspex would probably be more satisfactory. It permits the withdrawal of small quantities of sedimented material from the bottom upwards, the drops being deposited on a ruled slide held below the tap.

The faecal material, which is prepared as described later, is sedimented in a straight glass tube, 1 cm. in diameter and at least 4 ft. 6 in. long, at the bottom of which is fixed the tap. In the body of the tap, there is a hole C, 1 cm. deep and 1 cm. wide, which is filled with paraffin wax to a level just below the edge of the hole, thus leaving a small shallow cup in which the basal layers of the sediment accumulate. When sedimentation is complete, the tap is turned through 180° to the right until C is opposite the lowest hole in the barrel of the tap, D, which is slightly larger in diameter than C. The material caught in cup C is thus deposited down the spout on to a slide held just below. The tap is then turned back until C is below the end of the glass tube again, and the process is repeated if required until all the sediment has been examined. Each time C is turned back under the end of the glass tube an air bubble rises up, but in practice this has been found not to disturb the sediment to any great extent.

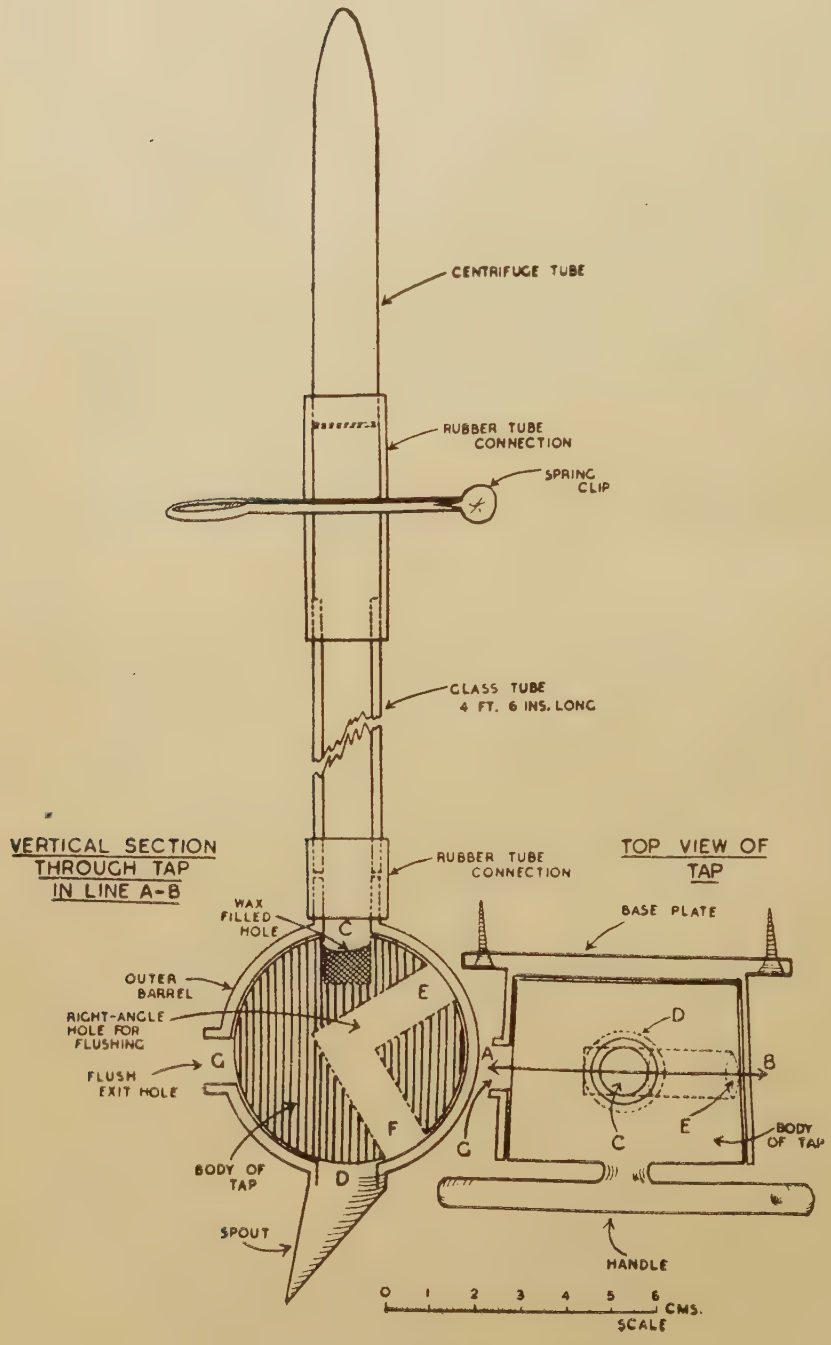
There is also a right-angle channel, E–F, bored through the body of the tap for draining the glass tube without disconnecting the tap. To do this the tap is turned to the right until limb F of the channel is beneath the end of the glass tube, and limb E is opposite the hole G in the outer barrel of the tap, when the contents of the glass tube will pour out of G. It is essential when constructing the tap to ensure that channel F is bored at a sufficient angle to the right-hand vertical edge of hole C; if this angle is insufficient, the tube will flush out when C is rotated to D.

The tap is secured to some firm support by screws through the base plate, and the glass tube is supported above the tap and connected to it by a collar of rubber tubing. Care should be taken to ensure that the end of the glass tube and the metal collar of the hole in the tap are in intimate contact to prevent eggs being caught up on any ridge. For this reason, the metal collar should be exactly the same thickness as the glass of the tube. At the top of the glass tube there is a short length of rubber tubing, of the same outside diameter as the glass tube, which can be closed by a spring clip. This rubber tubing should be sufficiently long to enable it to be bent down parallel to the glass tube.

METHOD OF USE

The method of using the apparatus for diagnosis or a fluke egg count is simple. One gramme of faeces is weighed out and thoroughly broken up with a glass rod in a suitable volume of water to which a few drops of a photographic wetting agent such as Burroughs and Wellcome's " Wettol " or Johnson's 326 have been added. This is important since *Fasciola* and paramphistome eggs stick readily to any smooth surface such as glass or metal even if vertical; the reduction of surface tension effected by the use of a wetting agent prevents this sticking, and renders much more easy the subsequent filtration and sedimentation of the eggs without loss. If a wetting agent is used the small drop of sediment caught in cup C runs easily and smoothly out of the spout when the tap is turned; if plain water only is used this drop will stick in the cup. These wetting agents have no effect on the eggs. The emulsified faeces are then strained through a 0·25 mm. mesh sieve, to remove the coarse faecal particles, and the filtrate centrifuged down in a hand centrifuge to a suitable volume with all the sediment collected in one tube only.

The glass tube is then filled to within 6 in. of the top with water also containing a few drops of wetting agent and the tap turned three or four times to make certain that drops caught in the cup are running satisfactorily out of the spout. The tap is then turned to the ready position with the cup under the end of the tube. Leaks should be avoided. The centrifuge tube is filled three-quarters full of water and the sediment stirred up. The rubber tube at the end of the glass tube is then bent over and the centrifuge tube fitted in. The spring


CENTRIFUGE TUBE
RUBBER TUBE CONNECTION
SPRING CLIP
GLASS TUBE 4 FT. 6 INS. LONG
RUBBER TUBE CONNECTION
VERTICAL SECTION THROUGH TAP IN LINE A-B
TOP VIEW OF TAP
WAX FILLED HOLE
OUTER BARREL
RIGHT-ANGLE HOLE FOR FLUSHING
FLUSH EXIT HOLE
BODY OF TAP
SPOUT
BASE PLATE
BODY OF TAP
HANDLE
0 1 2 3 4 5 6 CMS.
SCALE

clip is kept closed and the centrifuge tube is inverted two or three times to mix the contents. It is then held upside down as in the diagram and the spring clip opened so that the contents are precipitated suddenly into the water in the glass tube. The faecal material is left to sediment in the tube for at least one hour, after which time all the eggs will be found to have fallen into the cup, while the finer faecal material is still in suspension in the tube.

For counting purposes, the drop of sediment is run on to a glass slide with raised edges to prevent the liquid overflowing, and ruled in 0·5 cm. squares, each of which just fills the field of the lowest power of a binocular dissecting microscope. This magnification is sufficiently high for counting *Fasciola* or paramphistome eggs.

The translucent eggs are not always easy to see among the faecal débris which sediments at the same rate, and the addition of two drops of 1 per cent. neutral red to the sediment on the slide is a great advantage, water then being added until the volume in the hollow slide is just sufficient to spread out evenly without overflowing. The neutral red stains all the faecal particles a deep pink while the eggs remain unstained. If the slide is then examined under partial dark-field illumination (*i.e.*, with the microscope mirror just tilted off the optical axis) the eggs stand up as brilliant pin-points of light among the débris, and a slide can be counted easily in a matter of a minute or two. Fluke eggs present in a gramme of faeces are usually so few in number as to make a direct count difficult—in fact, seldom more than 500 per gramme have been found even in heavily infected animals. The sediment is examined drop by drop from the tap until no more eggs are present. In practice, however, it will be found that with a cup filled with wax to a certain depth, the first drop examined will contain an almost constant proportion of the eggs present in the whole gramme. This ratio can easily be calculated for any depth of wax filling, and once found, the count from the first drop only can be multiplied by this factor to give the total count in the whole gramme. After use the tap is turned to the draining position and the glass tube washed out.

Repeated counts on weighed amounts of faeces have given a high degree of consistency with this apparatus. It should be remarked, however, that both *Fasciola* eggs and the eggs of the common paramphistomes of ruminants in East Africa sink at the same rate in water, and in the case of mixed infections with these trematodes it is difficult except with practice to distinguish between the two sorts of eggs. There is a considerable overlap in the range of sizes and the only feature that is constant is the difference in colour. *Fasciola* eggs are usually yellowish, while those of the paramphistome flukes, when viewed by partial dark field illumination, are silvery and more translucent. Mixed infections are common in Kenya Colony, and confusion of the two kinds must be guarded against, particularly when making egg counts to determine the efficacy of treatment.

SUMMARY

An apparatus is described for the speedy separation of *Fasciola* eggs from a weighed amount of faeces.

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* *Meyer, E., and Arnold, L., Amer. J. Digest. Dis., 5:418*

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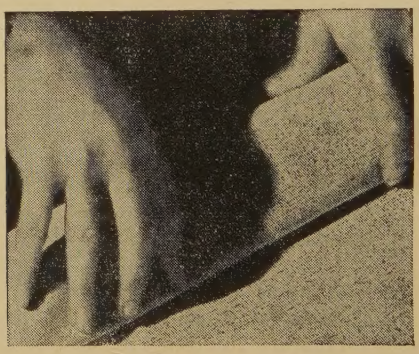

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